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A STUDY OF THE LIFE-HISTORY OF THE ONION FLY (*HYLEMYIA ANTIQUA*, MEIGEN)[1]

BY KENNETH M. SMITH, A.R.C.S.,

Adviser in Agricultural Entomology, Manchester University.

(With Plates X and XI.)

INTRODUCTION.

THE Onion-Fly has become a very serious pest of late years and is widespread throughout the country, more particularly in Lancashire and Cheshire where the observations recorded in this paper were made.

As in the cases of the Carrot and Cabbage Flies, the damage done by the maggots is all to the root and underground portions of the plant. In the larval condition, the food consists chiefly of onions but the maggot occasionally attacks shallots and leeks and has been recorded from Wales as feeding on tulip bulbs.

The pest is so plentiful in Lancashire that in certain districts, especially near large towns, it is impossible to grow onions at all.

SYNONYMS.

The Onion-Fly has been known under many names. The various synonyms include the following:

Phorbia ceparum, Meig.
Phorbia cepetorum, Meade
Pegomyia cepetorum
Pegomyia ceparum
Anthomyia ceparum
Anthomyia antiqua
Hylemyia antiqua, Meig.

The synonyms most commonly in use at present are *Phorbia cepetorum*, Meade, and *Hylemyia antiqua*, Meig. In his *Descriptive List of the British Anthomyiidae*, Meade gives the fly the name *P. cepetorum* and describes quite another species under *H. antiqua*. The *Palaearctic Catalogue of Diptera* and Stein's recent Monograph of the *European Anthomyiidae*, however, give the name as *Hylemyia antiqua*, Meig. It is, therefore, likely that Meade mis-identified this fly or described the same species twice.

It will be more correct in the future to refer to it under the name of *Hylemyia antiqua*, Meig.

[1] A grant in aid of publication has been made for this communication.

Life-History.

The Egg. Description. The egg of the Onion-Fly is white in colour and 1 mm. in length. The outer coating is ridged and there is a shallow depression down one side extending about a third of the distance. The egg much resembles that of *Chortophila brassicae*, the Cabbage Root Fly, except that it is larger and the depression is shorter and shallower than is that in the egg of the Cabbage-Fly. Pl. X, fig. 1 shows the egg of the Onion-Fly (A) compared with the egg of the Cabbage Root Fly (B).

Duration of Egg Stage. This period varies according to the temperature. The usual time is about three days but is occasionally prolonged to six or seven days.

The Larva. Description. On hatching from the egg, the young maggot makes its way through the soil and attacks the root of the onion, boring its way in through the base of the bulb. The full-grown larva and the newly hatched larva do not differ materially except in size.

When full grown the maggot is from 9–10 mm. long and $1\frac{1}{2}$ mm. broad at the thickest part. It is white in colour, flattened at one end and tapering to a point at the other. At the broad flattened end which is the "tail," are numbers of tubercles arranged as shown in fig. 2. In the centre of the flattened end are two chitinous projections, these are the posterior spiracles or "breathing pores." Anteriorly, at the "head" end, is a pair of black hook-like "jaws" of strong chitin by means of which the larva bores its way into the onion. These "jaws" are continuous with a chitinous framework to which are attached a number of muscles; surrounding the hooks on the outside of the "head" is a pair of large fleshy lip-like structures. There is also a pair of small papillae. A little further back are the anterior spiracles, these consist of two flattened fan-like outgrowths, one on each side. Each spiracle is composed of eleven finger-like lobes, this number is not constant but varies in different larvae. Fig. 3 is a drawing of the anterior end of the adult maggot and fig. 4 is an enlarged photograph of the whole insect.

Length of Larval Period. From a number of observations made on the length of the larval stage, it was found that the periods ranged from eighteen to twenty-seven days, the average being twenty days. This was in green onions; according to Severin and Severin (1) the larval period is prolonged into four or five weeks in seeded onions of the previous year. Larvae of the later generations living in larger and more mature onions seemed to take longer over that stage than the first generation.

Pupation. When fully grown, the larva leaves the onion and enters the soil to transform into the pupal condition. The exact position in the ground varies but is generally at a depth of two or three inches and may be close up against the onion or a short distance away. On pulling up

an attacked plant the pupae may usually be found in the cavity thus created.

Description of Puparium. The puparium is oval in shape, dark brown in colour, occasionally varying to a lighter colour, and 6 or 7 mm. in length. The larval structures are retained and can easily be made out. Fig. 5 is a photograph of the puparium.

Duration of Pupal Period. The following observations were made with numbers of larvae in order to determine the time occupied by the pupal stage.

Larvae pupated	Adult flies hatched	Period
June 20th	July 9th	19 days
„ 23rd	„ 10th	17 „
„ 25th	„ 12th	17 „
„ 29th	„ 16th	17 „
„ 29th	„ 15th	16 „

This gives approximately an average of seventeen days for the length of the pupal period.

Description of Adult Fly. The male is a grey insect somewhat like a house-fly in appearance, though rather lighter in colour. Its body is about 6 mm. long and measures ½ inch across the wings. The thorax is of a lighter grey than the rest of the body and has a number of large bristles interspersed with small ones running longitudinally giving the thorax a banded appearance. The abdomen is darker than the thorax and is much more heavily set with black bristles; there is a band of paler grey down the centre of the abdomen. In the male the eyes are very closely set together. The female is very similar to the male in general appearance, except that it is rather lighter in colour, the eyes are widely separated and the abdomen is broader and pointed at the end, owing to the presence of the ovipositor.

The following description of the Onion-Fly under the name *Phorbia cepetorum* is quoted from Meade's *Descriptive List of the British Anthomyiidae.*

"*Head:* face slightly prominent; epistome flat; eyes of male contiguous; antennae of moderate length with the arista thickened and pubescent at its base, but nearly bare in the middle and at the extremity.

Thorax: with the scutellum of a light yellowish-grey colour; the former marked with four indistinct pale brown stripes, and with four rows of black bristles.

Abdomen: oblong and rather narrow, cinereous, clothed with black hairs and showing silvery white reflections when viewed from behind; it is marked down the dorsum with a row of elongated narrow triangular black spots, which form a sub-continuous stripe; the anal segment is grey, small and rather pointed; the sub-anal male appendages are large and hairy.

Wings: hyaline, with the third and fourth longitudinal veins nearly parallel to each other, and the external transverse ones straight, and a little oblique; *Calyptra* and *Halteres* both pale yellow.

Legs: sometimes piceous; hind femora almost bare of hairs or bristles at the base of their under surfaces; hind tibiae of the males furnished with a few short bristles along the middle and upper part of their inner sides. The female is very similar in colour to the male; the eyes are widely separated, the intervening space being red at its front part; the abdomen is dull grey mostly immaculate, conical and pointed at the apex; the calyptra are white and the halteres yellow."

Fig. 6 is a drawing of the female Onion-Fly.

Length of Life of Adult Fly. The writer was unable to determine the length of life of the adult flies under natural condition. In the laboratory, however, they showed considerable longevity. At room temperatures and fed on casein, the flies lived for periods ranging from three weeks to two months.

This may be partly due to the artificial conditions of feeding and the absence of natural enemies, etc. In this connection it is worth mentioning that the flies refused to feed upon sugar and water in captivity but fed readily upon casein.

This is curious when it is considered that the "poisoned bait" method of control, which consists of poisoning the flies with molasses and sodium arsenite, is so largely used. This possibly may be explained by the difference in the sugars used.

Development and Number of Generations. From observations made during the summers of 1920 and 1921, both in the field and in the insectary, it appears that there are three generations of *Hylemyia antiqua* in a season, the third being incomplete. There is no well-marked division between the broods but each one overlaps the other, so that maggots in all stages, puparia and adults are found throughout the summer.

In the unusually hot autumn of 1921, the larvae of the third generation were found attacking autumn sown onions, quite late in October.

The adult flies hatched from overwintering puparia were first noted on the wing on May 8th, though odd specimens have been known to hatch in a mild winter as early as January 25th.

The flies were first observed in the onion fields on May 30th and 31st and in much larger numbers during the early days of June. The maggots of the first generation commenced hatching on the second of June. These had mostly pupated by the end of the month. Flies of the first generation commenced hatching at the beginning of the second week in July and second generation adults were seen ovipositing on August 24th.

The larvae of the third generation have usually pupated by the end of September or the beginning of October though in late seasons they may continue to feed till the end of the latter month.

The third generation thus winter as puparia and the adult flies emerge in the following spring.

Taking forty days as a fair estimate of the duration of the life-cycle from egg to adult, the following table gives an idea of the approximate times of appearance of the generations.

1st Generation	May 28th	→ Eggs deposited by flies emerged from overwintering puparia
	June 1st	→ Larvae of 1st generation hatch
	June 19th	→ Larvae of 1st generation pupate
	July 8th	→ Adult flies of 1st generation emerge
		Approximate Time for Maturation of Flies = 7 *days.*
2nd Generation	July 15th	→ Eggs deposited by 1st generation adults
	July 18th	→ 2nd generation larvae hatch
	Aug. 5th	→ 2nd generation larvae pupate
	Aug. 24th	→ 2nd generation adults emerge
		Maturation Period = 7 *days.*
3rd Generation	Sept. 1st	→ Eggs deposited by 2nd generation adults
	Sept. 4th	→ 3rd generation larvae hatch
	Sept. 22nd	→ 3rd generation larvae pupate and hibernate

It should be understood that this table is entirely artificial and does not attempt to do more than give approximate dates for the various appearances. It should also be made clear that the generations are not sharply divided off as they appear in the diagram. Some first generation adults may still be emerging at the same time as the second generation adults; and first and second or second and third generation larvae are often found together.

Habits. Food Plants. The food consists mainly of the onion; the maggots, however, occasionally attack leeks and shallots and have been recorded as feeding upon tulip bulbs and lettuce. Under experimental(1) conditions the larvae have been induced to complete their development in fresh manure and also in radishes.

Injuries Produced. The worst damage to the onion occurs in the spring when the plant is still a seedling. Owing to the small size of the onion and the large numbers of maggots produced, the young plants are devoured wholesale, the larvae migrating from onion to onion, leaving nothing but the green portion above ground. As the onion increases in size, symptoms of attack are yellowing and wilting of the tops which

finally lie prone on the ground, the bulb in bad cases being reduced to a rotting semi-liquid mass. Any number of maggots from 3 or 4 to 25 or 30 may be found in one onion bulb.

The maggot, as a rule, enters the onion at the base and works its way upwards, occasionally, however, it enters at the side. Pl. XI, fig. 7 shows onion bulbs destroyed by the maggots.

Reproduction. Oviposition. The eggs are laid on the onion plant, in clusters of half a dozen or more and sometimes as many as twenty or thirty may be found together. They are deposited usually under the thin sheathing leaf surrounding the stem, or in the crutch formed by the outside leaf and the stem. Occasionally the eggs are deposited in cracks in the soil but the more usual procedure is to lay them on the plant itself. The attachment is very slight and eggs found on the surface of the soil beneath the onion have usually been detached by some external agency such as rain or wind.

Pre-oviposition Period. This is an important phase in the life of the Onion-Fly and more so in the light of recent attempts to control this pest by means of poisoned bait intended to kill the fly during the oviposition period.

Sanders (2) puts this period at ten to fourteen days, while Severin and Severin (1) give twelve to sixteen days as the time.

This is a point difficult to determine with any great degree of accuracy owing to the probable effects of the artificial conditions of captivity upon the development of the fly.

From dissections of flies in the laboratory at varying periods from the time of emergence, the writer is inclined to put the time of maturation at a week and sometimes as long as nine or ten days.

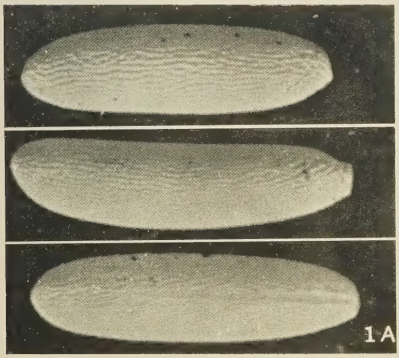
Hibernation. The usual method of hibernation is undoubtedly in the pupal condition. There are cases on record, however, which show that the larvae are also capable of passing the winter.

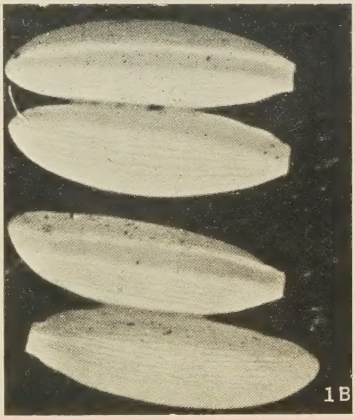
According to some authors (3) the insect hibernates as an adult but confirmation of this fact is lacking.

Parasites. One Hymenopterous parasite belonging to the order Braconidae was bred from Onion-Fly pupae, the species being *Aphacreta cephalotes*. This parasite was responsible for largely reducing the numbers of the later generation of Onion-Flies in the summer of 1920. As many as fourteen fully developed adults were dissected from one pupal case of the Onion-Fly.

The parasite also attacks the larvae of *Psila rosae*, the Carrot-Fly. Fig. 8 is a drawing of *Aphacreta cephalotes* and fig. 9 its pupa.

Another useful natural enemy is a beetle *Aleochara bilineata* belonging to the order Staphylinidae or "Rove" Beetles. The larva of this insect





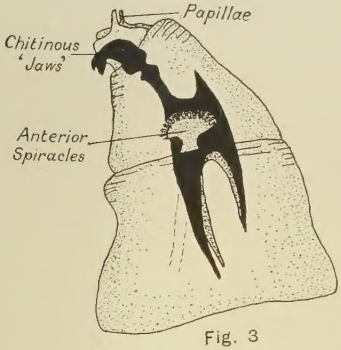


Fig. 3

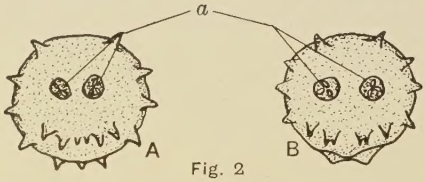


Fig. 2

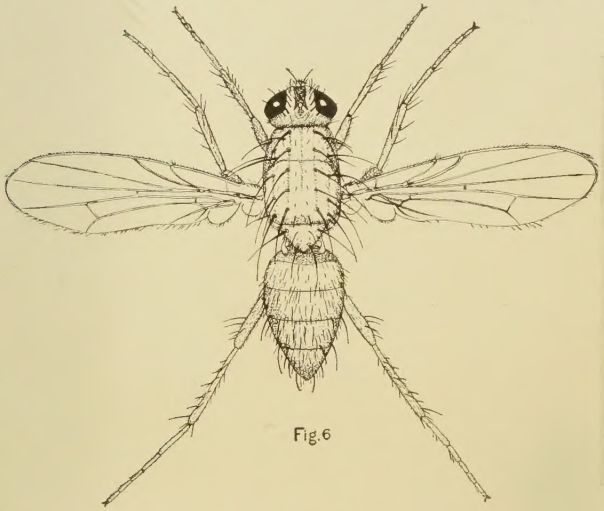


Fig.6

7

Fig. 8 *Ovipositor*

Fig. 9

10

is predaceous upon the pupae of the Onion-Fly, of the Cabbage Root Fly and allied species. This larva bores its way through the hard shell of the pupal case and feeds upon the pupa inside, it then completes its development in the case, emerging later on as the adult beetle. Fig. 10 is a photograph of *Aleochara bilineata*.

Methods. The Onion-Flies were studied in the field, in the open-air insectary and in the laboratory; for the two latter methods large glass cylinders were employed. These were placed over onions growing in pots and the tops were covered with fine muslin. This allowed a clear view of the insects confined within.

Acknowledgments are due to Mrs Tattersall for her kind assistance in preparing the drawings.

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(2) SANDERS, J. G. *Journal of Economic Entomology*, VIII. 89.

(3) SMITH, J. B. and DICKENSON, E. L. (Feb. 12, 1907). *New Jersey Agricultural Experiment Station, Bulletin* 200.

EXPLANATION OF PLATES X AND XI

PLATE X

Fig. 1 A. Eggs of *Hylemyia antiqua* compared with

Fig. 1 B. Eggs of *Chortophila brassicae*.

Fig. 2. Posterior spiracles. A. Caudal end of onion maggot. B. Caudal end of cabbage root maggot. (*After* Gibson and Treherne.)

Fig. 3. Anterior end of onion maggot, showing spiracle and chitinous 'jaws.'

Fig. 4. Full grown onion maggot.

Fig. 5. Puparia of onion-fly. Dorsal and ventral aspects.

Fig. 6. Female onion-fly.

PLATE XI

Fig. 7. Onions showing damage caused by the feeding of the maggots.

Fig. 8. *Aphacreta cephalotes*. A parasite of the onion-fly.

Fig. 9. Pupa of *Aphacreta cephalotes*.

Fig. 10. *Aleochara bilineata*, a beetle whose larva is predaceous upon the pupa of the onion-fly.

All except Fig. 7 much enlarged.

(*Received February 9th*, 1922.)

THE SMUT OF NACHANI OR RAGI (*ELEUSINE CORACANA* GAERTN.)

By G. S. KULKARNI.

(With 2 Text-figures.)

This smut was first observed by the writer in 1918 at Malkapur in the Kolhapur State. Later it was collected in the districts of Surat, Nashik, and Ratnagiri in the Bombay Presidency.

The disease is visible only in scattered grains in the head, the majority of grains developing normally. Sometimes the affected grains are single, sometimes grouped in patches of varying size, frequently confined to one side or towards the base or apex of the head.

The sori occur in the ovary as round or occasionally elongated bodies. These project beyond the glumes and they may be from one to six times the diameter of the normal grains (Fig. 1), being often 3–8 mm. in diameter when round, and 4–15 mm. in length when elongated. When fresh their colour is green, occasionally pinkish, but they turn chocolate-brown or dirty black on drying. The colour is due to the membrane, the spore mass being always deep brown to black. On rupture of the membrane the inside is found to contain a powdery black spore mass. The spores are round, 6·6–12·10μ in diameter, dark brown, and have spiny walls.

Germination of the spores occurs easily in nutritive solutions (*e.g.* tomato broth). The spore puts forth a thick, colourless, septate promycelium, and forms spindle shaped sporidia which bud very freely (Fig. 2).

The life-history of the fungus was studied in order to determine whether the disease was seed-borne. A small quantity of Nachani seed was infected with the spores of the smut and was divided into two lots, one lot being then treated with 2 per cent. copper sulphate solution for 10 minutes. The two lots were then sown in separate plots. Smut appeared on a few plants in the plot raised from the infected seed, while in the treated plot all the plants were free from the smut. It appears therefore that the smut is seed-borne and is amenable to copper sulphate seed treatment.

Fig. 1.

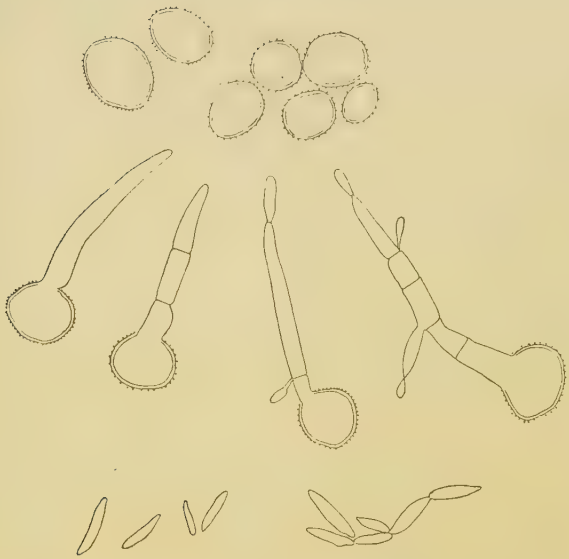


Fig. 2.

The study of the germination of the spores of the smut shows it to be a species of Ustilago, and as no smut of Nachani has been recorded the name *Ustilago Eleusinis* has been proposed, and the following description is given both in English and Latin.

Ustilago Eleusinis nov. sp.

Sori scattered, green or pinkish at first, later becoming darker. Spore mass powdery. Spores round, 6·6–12·10 μ in diameter and spiny. Promycelium hyaline, septate, giving rise to many spindle-shaped sporidia.

Habitat: on *Eleusine coracana* at Malkapur in October 1918 in the Bombay Presidency, India.

Ustilago Eleusinis nov. sp.

Sori sparsis, primum viridibus vel roseis, dein fuscescentibus; sporarum massa pulveracea; sporis globosis, 6·6–12·10 μ diam. echinulatis; promycelio hyalino septato, sporidiola numerosa fusiformia emittente.

Hab. in ovariis *Eleusinis coracanae* ad Malkapur, in provincia Bombayensi Indiae, Oct. 1918.

(*Received March 4th*, 1922.)

ON THE YOUNG LARVAE OF *LYCTUS BRUNNEUS* STEPH.

By A. M. ALTSON, F.E.S.

(With 2 Text-figures.)

CONTENTS.

Introduction.

This paper describes the first and second instar larvae of *L. brunneus*, with some observations on their habits, and it includes a few notes on certain parts of the anatomy of the larvae of later instars.

These observations and notes were made, partly in 1920 and in the following year, during an investigation into the ravages of the beetle, and constitute part of the results, of which some have been published elsewhere.

No account of the early larval stages of any beetle of the genus *Lyctus*, or of the family *Lyctidae*, appears to have been published.

In another paper[1] a description is given of the position of the young larva at the time of maturation, and the observations here are continued from that point.

Description of First Instar Larva[2].

The first instar larva (Text-fig. 1, 1) is creamy white and is very small, averaging 0·65 mm. long by 0·23 mm. wide at the thorax. It is sub-

[1] "The method of oviposition and the egg of *Lyctus brunneus* Steph." (*In the press.*)

[2] Nomenclature after Hopkins (1909, Tech. Ser. No. 17).

cylindrical and its body is straight and not arched as the later instars are[1].

The following description is based on specimens mounted in balsam, or glycerine.

The head (Text-fig. 1, 2 A) is broader than thick and is circular viewed dorso-ventrally. It is partially enveloped by the pro-thoracic folds. There are a pair of rudimentary eyes (*e*) composed of pigmented spots and situated below and posterior to the antennae (*a*). No consistency in the shape of the eyes was observed, the number of pigmented spots vary, the majority are in juxtaposition, but a few are some distance apart, and they are deep purple in colour.

(Eyes were found on the larva of each instar[2]. Dugés [1883] in his description of—apparently—the full-grown larva of *L. carbonarius*, refers to a pair of protuberances which he considered to be eyes, and which he figured between the mandibles and antennae. This position differs from that of those of *brunneus*.)

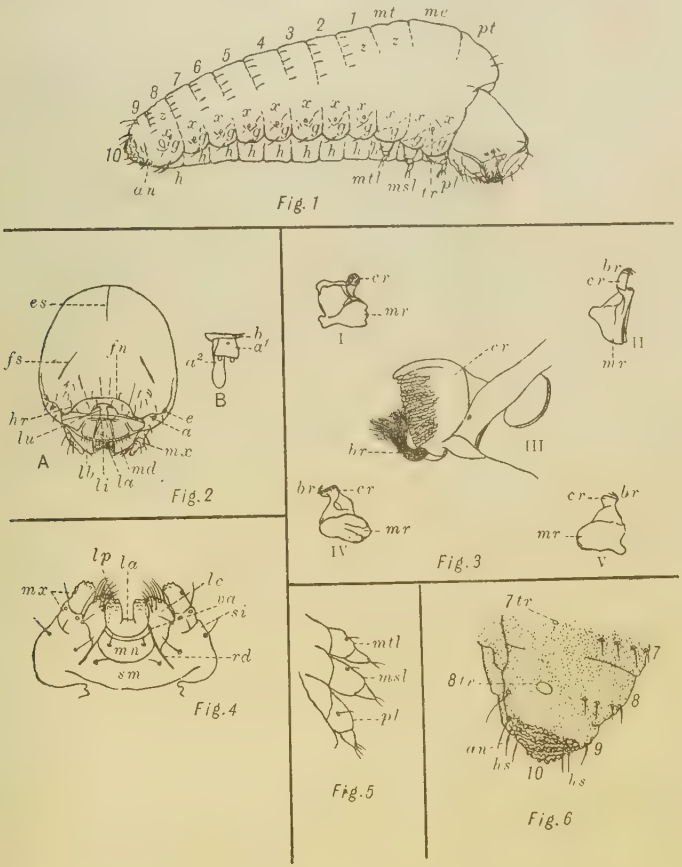
The antennae (Text-fig. 1, 2 B) are telescopic and are situated in recesses and consist of one basal joint (*b*) and two apical pieces (a^1, a^2). One apical piece (a^1), which is the antenna proper, is wider at its apex than its base and terminates in two minute fleshy protuberances, towards its base is a sensory pit. The other apical piece (a^2) is venter and is longer than the dorsal piece. (A ventral apical piece has been found venter to the apical joint of the antenna proper in every instar[2]; in the later stages it decreases in size in an inverse ratio to the size of the apical joint of the antenna, until in the full-grown larva it is barely one-sixth the length.)

The mandibles (Text-fig. 1, 3) are of the same type in each instar and are of a peculiar structure. The molar or distal joint (*mr*) is roughly triangular in outline dorso-ventrally; it is tridentate (Text-fig. 1, 3 IV). Situated dorso-posteriorly and above the molar is an extended dorsal condyle[2], which terminates in a small rounded structure (*cr*) serrated on its inner lateral face; and arising from the outer lateral face of the serrated structure, is a group of chitinised setae (*br*), which are curved round the posterior border to the inner lateral face (Text-fig. 1, 3 III). This extended dorsal condyle which does not appear to bear any relation to the movable *prostheca* of Kirby and Spence (Packard, 1909), is situated at the end of the hypo-pharynx. It comes into operation when the molars are opened

[1] Gahan (1920) in describing the first instar larva of *Anobium punctatum* De Geer states: "At this time they are...straight-bodied, instead of having the body strongly curved as in the older larvae;...." This similarity between these first instar larvae of these systematically closely related beetles, is of some interest.

[2] This is not mentioned by Munro (1915–16) in a description of the full-grown larva.

and in the act of biting, and their function is, apparently, crushing the food—in the later instars the particles of wood tissue—whilst the setae strain or align it, before it passes into the pharynx.



Text-fig. 1.

The labrum (Text-fig. 1, 2 A) is clearly defined and is sparsely fringed with very fine bristles. The clypeus and epistome were not discernible, but the anterior border of the frons (*fn*) is strongly defined and appears as a stoutly chitinised semi-circular rod (*hr*) supporting the upper region of

the head. Just below this support is a small semi-circular piece of chitin (*lu*) forming the lumen in which the crushing organ of the mandibles work; between this piece and the anterior border of the frons, a few setae are found symmetrically arranged on either side. The frontal (*fs*) and epicranial (*es*) sutures were barely visible.

The maxillae (Text-fig. 1, 4) consist of two parts, an outer double-jointed palp (*mx*), and an inner piece—the lacinial lobe (*lc*). The stipes (*si*) is present, but the cardo was not distinguishable. The maxillary palp is telescopic, and bears a few scattered setae and sensory pits; the apical joint terminates in five fleshy protuberances. The lacinial lobe bears several stiff setae towards the apex; it is dorsal to the maxillary palp and fused to it; lying venter is the labium (*la*), which is partially defined by chitinised rods (*rd*) supporting it and the maxillae. The labium consists of two single-jointed palps (*lp*) arising from a broad fleshy base, the vaginant membrane (*va*), whose inner surface forms the ligula (*li*), which bears scattered fleshy protuberances; there are a few of these on the apex of each labial palp. The mentum (*mn*) and sub-mentum (*sm*) are clearly defined.

The thorax (Text-fig. 1, 1) is well developed. The pro-thorax (*pt*) partly envelopes the head; and bears a pair of spiracles (*tr*). Each thoracic segment consists of scutal (*x*), scutellar (*z*), epi-pleural (*g*), and sternal (*h*) lobes. Each segment bears a pair of three jointed legs (Text-fig. 1, 5); the pro-thoracic pair (*pl*) are more strongly developed than the others, and terminate in a strong seta surrounded by three longer bristles; the meso- (*msl*) and meta-thoracic legs (*mtl*) each bear two bristles towards their apex.

The abdomen (Text-fig. 1, 1) consists of ten segments (1–10). The first eight are composed of scutal (*x*), scutellar (*z*), epi-pleural (*g*) and sternal (*h*) lobes. There is one bristle on each epi-pleural and a lateral row of four on each scutellar fold towards the anterior border. On each of these segments are a pair of spiracles, those of segments 1 to 7 being of uniform size, whereas the pair on the 8th segment are approximately six times as large as the others; a peculiarity which Perris (1876) considered—in reference to the full-grown larva of *L. linearis* Goeze (*canaliculatus* Fab.) —serves to distinguish the larva of *Lyctus* from all others. No pre-scutal lobes were observed on the abdomen. The 9th and 10th segments (Text-fig. 1, 6) consist of a series of fleshy protuberances functioning as an anal foot; with the exception of a scutal lobe on the 9th segment, no other lobes were discerned. The anus (*an*) is situated on the 10th segment and is partly enveloped by the hind margin of the 9th segment.

There are several large setae symmetrically arranged on the 9th and 10th segments, which act as "hatching spines" (*hs*).

The larval integument of the thorax and abdomen appears minutely punctured (Text-fig. 1, 6).

Description of Second Instar Larva.

The following description—based on the examination of balsam mounts—is comparative and only points of difference with the first instar larva are referred to.

The second instar larva (Text-fig. 2, 1) is similar in appearance to the later stages, that is, it is arched. It is subcylindrical, and is creamy white in colour, except towards the apex of the abdomen dorsally, where the alimentary tract filled with wood tissue gives it a coloured area, the colour depending upon that of the wood on which it has been feeding. The chaetotaxy is more complex than in the first instar.

The head (Text-fig. 2, 2 A) is slightly rounded from a lateral aspect. The frontal (*fs*) and epicranial (*es*) sutures are fairly pronounced. The antenna (Text-fig. 2, 2 B) differs from that of the first instar, the apical dorsal piece (a^1) is longer than the venter piece (a^2). The trophi are of the same type with a variation in the number of and arrangement of the setae. The eyes (*e*) are more pronounced. The epistomal and clypeal sutures were not discernible.

The thorax (Text-fig. 2, 1) is clearly defined. Each segment is composed of scutal (*x*), scutellar (*z*), prescutal (*o*), epi-pleural (*g*), and sternal (*h*) lobes. The apical joint of the pro-thoracic leg (*pl*) bears six bristles and the terminal seta (Text-fig. 2, 3); the apical joints of the meso- (*msl*) and metathoracic legs (*mtl*) bear three bristles and a terminal seta.

The abdomen (Text-fig. 2, 1). Segments one to four are composed of scutal (*x*), scutellar (*z*), prescutal (*o*), epi-pleural (*g*), and sternal (*h*) lobes; segments five to eight of scutal (*x*), scutellar (*z*), epi-pleural (*g*) and sternal (*h*) lobes. No row of setae was found on segments one to four, but these are present on five to eight. Each epi-pleural fold bears two setae. The 9th and 10th segments (Text-fig. 1, 4) do not bear any fleshy protuberances and no setae appear on the last segment.

The integument of the thorax and abdomen is covered with symmetrically arranged rows of minute chitinised scales (Text-fig. 2, 4).

Observations on the Larvae.

First Instar. As soon as the young larva is fully developed, it commences feeding upon the residual-yolk-mass whilst still enclosed within the chorion, which is soon broken at the posterior pole by its

movements and its "hatching spines." Through the aperture thus caused the first particles of excrement pass into the vessel. As the larva progresses it gradually fills the chorion with excrement.

The larva is now travelling along the vessel towards the point of

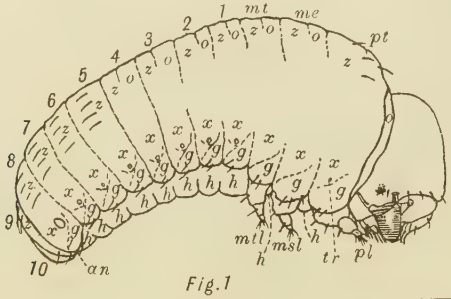


Fig. 1

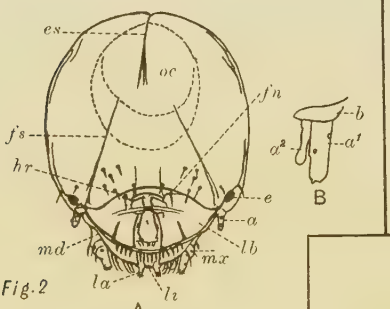


Fig. 2

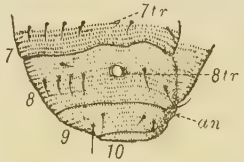


Fig. 4

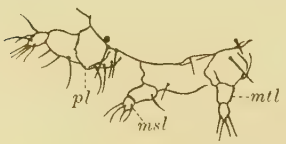


Fig. 3

Fig. 5

Fig. 6

Text-fig. 2.

access of its parent's ovipositor. By the time it has eaten its initial food, it has nearly increased in girth enough to fill the vessel, and is able to obtain a grip upon its walls and the necessary purchase—aided by the anal process—to enable it to start attacking the walls and contents of the vessel.

At the time of maturation, the mandibles are pale brown and gradually darken as the residual-yolk-mass is consumed, and harden to enable the larva to commence its attack.

In thirteen specific instances it took the young larva from three to five days to consume the residual-yolk-mass. Very little of the vessel contents is eaten, but apparently enough to clear a space for itself to undergo an ecdysis; which was observed to occur between seven and ten days after maturation. Shortly after settling down in the vessel preparatory to moulting, the head capsule is exserted, and the thorax and abdomen become slightly arched.

Second Instar. After extricating itself from the first instar exuvium, which splits primarily along the frontal and epicranial sutures and then along the thorax, the larva rests to harden.

About 24 hours later it commences to bore into the wood tissues.

In the majority of cases observed, the young larva had eaten its way through the vessel, into the tissues, at approximately right angles to its original path, and had taken a downwards course for some distance before again turning at right angles; it had then started boring in a direction opposite to that in which it had originally begun travelling.

In the others, the larva had struck off to the left or right downwards, the direction depending upon which outer margin of the piece of wood the egg had been deposited in. In these cases the larva also turned again after boring some distance down.

In a few instances it was noticed that after biting through the vessel in which it was hatched; it had come into an adjacent vessel, and had turned into this and utilised it for some distance until it had gradually enlarged it and worked its way into the tissues surrounding the vessel.

The foregoing observations are based upon the behaviour of second instar larvae in small pieces of mahogany, of a size which enabled the writer to find the eggs after each piece had been made accessible to females for one night.

The sizes of these pieces, which were split on all faces longitudinally with the vessels, and cut transversely at the ends, ranged from one to two inches in length by one-eighth to about three-eighths of an inch in width and thickness. So that the larvae's movements were considerably confined.

No doubt, in large pieces of wood such as boards, baulks, etc., the larvae would not return in a direction opposite to that in which they had originally travelled along the vessel—after once boring into the tissues—unless they found themselves at the extreme edge or surface of the wood; but would bore in the same direction, only in a lower plane.

The wood tissues, which constitute the food of the larva, are, after passing the crushing apparatus referred to, further broken up in the proventriculus. This consists of longitudinal rows of short chitinised setae. In the full-grown larva there are eight rows (Text-fig. 2, 5, 6), four superior and four inferior.

The proventriculus, oesophagus, pharynx, and the trophi are cast at each ecdysis.

It was observed that the larva's method of boring is partly assisted by the following. (*a*) Its habit of packing the frass behind it into a compact mass by means of continual pressure of the curved apex of the abdomen against the frass; in this, it is aided by the wood tissue contained in its convoluted proctodeum. (*b*) By slightly revolving as it bores, thus enabling it to bite out a bore which is circular in transverse section and in more or less the one plane. (*c*) Its possession of a large quantity of body fluid, which flows rapidly under control and functions in a manner somewhat similar to the body fluid of an emerging Cyclorhaphous Dipteron.

The legs were seen to be used to assist it in revolving, and for clearing out particles of wood tissue, or the frass of another bore into which it had struck. The dorso-lateral thoracic region was observed to fit closely to the bore when distended by the body fluid, but the legs were free to move within the cavity formed by the lateral epi-pleural lobes and the sternum.

Conclusions.

The rudimentary compound eyes, which are present in all the larval stages of *L. brunneus*, are most clearly defined in the first and second instars. Their presence being probably due to their existence in the remote free-living ancestral larva.

The retention and value of them to a wood-boring larva at first appear obscure, but, when it is remembered that the young first instar larva works its way along the vessel towards the point of access of its parent's ovipositor, to consume the residual-yolk-mass, it is coming towards light, and the surface of the wood; the value of its eyes is obvious. The larva is helpless on the surface of the wood. (None were ever able to get back into a vessel after being placed on the surface.)

In the second instar the value and the use of its eyes are clearly demonstrated by the observations in the foregoing account of the second instar larva's behaviour in the small pieces of wood used in the breeding experiment. For, when it found itself in a vessel at the side it always

bored down and towards the centre of the piece, or if in a vessel at either end it bored down and turned on a lower level towards the centre. It is apparent that the rudimentary eyes enable the larvae to remain within the wood. The larva can be said to be negatively heliotropic.

Summary.

1. At maturation, the first instar larva commences to feed upon the residual-yolk-mass contained in the anterior part of the egg, remaining within the chorion to do so. It takes three to five days to accomplish this. It sometimes eats a few particles of the walls or contents of the vessel before settling down to moult.

2. From seven to ten days after reaching maturity the young larva undergoes an ecdysis and then commences its boring operations in the wood.

Acknowledgments.

The investigation, of which this paper records part of the results, was suggested by Prof. H. Maxwell-Lefroy, Imperial College of Science, to whom the writer has to express his thanks; and to the Committee of the Scientific and Industrial Research Department, for a grant to carry on the work.

The writer is also indebted to Dr C. J. Gahan, Keeper of the Department of Entomology, Nat. Hist. Mus., for identifying specimens of *L. brunneus*; to Prof. Percy Groom, Imp. Coll. Sci., for identifying the various species of timber used in this work.

In addition, thanks are due to Dr A. D. Imms, Rothamsted Experimental Station, for his advice, and assistance in connection with the publication of this paper; and to Prof. S. MacDougall for his efforts to get the original paper published as a whole.

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EXPLANATION OF TEXT-FIGURES 1 AND 2

REFERENCE LETTERING.

a, antenna; a^1, a^2, apical joints of antenna; *an,* anus; *b,* basal joint of antenna; *br,* brush-like group of setae on crushing organ; *cr,* crushing organ of mandible; *e,* eye; *es,* epicranial suture; *fn,* frons; *fs,* frontal suture; *g,* epipleural lobe; *h,* sternal lobe; *hr,* semicircular rod of chitin supporting upper region of head; *hs,* hatching spines; *la,* labium; *lb,* labrum; *lc,* lacinial lobe; *li,* ligula; *lu,* piece of chitin forming lumen for crushing organs; *md,* mandible; *me,* mesothorax; *mn,* mentum; *mr,* molar or distal joint of mandible; *msl,* meso-thoracic leg; *mt,* metathorax; *mtl,* metathoracic leg; *oc,* occipital foramen; *pl,* pro-thoracic leg; *pt,* pro-thorax; *rd,* rod; *si,* stipes; *sm,* submentum; *tr,* spiracle; *va,* vaginant membrane; *x,* scutal lobe; *z,* scutellar lobe; 1–10, abdominal segments.

TEXT-FIG. 1.

1. First instar larva. Camera lucida ×80.
2. (A) Dorsal aspect of head. C.l. ×128.
   (B) Lateral aspect of antenna. C.l. ×177.
3. Mandibles: (I) Latero-anterior; (II) Dorsal; (III) Crushing organ and brush of full-grown larva; (IV) Inner lateral; (V) Outer lateral. C.l. ×132.
4. Ventral aspect of maxillae of 2nd Instar larva. C.l. ×177.
5. Legs of larva, left. C.l. ×132.
6. Apex of abdomen, segments 7–10. C.l. ×132.

TEXT-FIG. 2.

1. Second instar larva. Camera lucida ×80.
2. (A) Dorsal aspect of head. C.l. ×128.
   (B) Lateral aspect of antenna. C.l. ×177.
3. Legs of larva, left. C.l. ×132.
4. Apex of abdomen, 8–10, and part of 7. C.l. ×128.
5. Transverse section of proventriculus of full-grown larva. C.l. ×80.
6. Optical longitudinal section of proventriculus of full-grown larva. Drawn from photomicrograph. ×80 (approx.).

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EFFECT OF HIGH ROOT TEMPERATURE AND EXCESSIVE INSOLATION UPON GROWTH.

By WINIFRED E. BRENCHLEY, D.Sc.

(*Rothamsted Experimental Station.*)

ASSISTED BY

KHARAK SINGH, M.A.

(*Punjab Agricultural College, Lyallpur.*)

(With 2 Text-figures.)

In an earlier paper(3) it was demonstrated that the reduction of light due to the over-crowding of barley plants brings about a condition of light starvation which has a harmful effect upon growth, even when an abundance of food and water is supplied to the roots. The suggestion was made that this factor of light competition might be equally or even more important in the case of broad-leaved plants, as greater overshadowing might occur.

To test this water culture experiments were repeated several times with peas at different seasons, 64 plants being closely crowded in a solid square, and 64 others having abundant room to prevent any shading of one another. The nutrient solutions were changed frequently and the tendrils of the peas were cut off as early as possible to prevent damage from one plant clinging to its neighbour when being moved.

Sutton's[1] Harbinger peas were used throughout.

In a test carried on from September 10th to December 21st, 1920, the prevailing conditions were:

Average weekly maximum temperature of house	9–26° C.
,, ,, minimum ,, ,,	2–11° C.
Total hours of sunshine per week	45·9–48

Temperature and sunlight both fell off considerably during the latter half of the experiment.

[1] We are indebted to Mr Martin Sutton for the gift of all the seeds used in these experiments.

From a comparatively early date the advantage seemed to be with the spaced plants, and became more marked as growth proceeded and the intensity of light decreased with the waning season. The difference in shoot growth was not noticeable for several weeks, but the roots of the spaced plants soon became strong and bunchy, being larger than any of the crowded roots. In the latter the roots on the outside were comparatively strong, but decreased steadily in size towards the middle of the square, where they were fairly long but very thin. At harvest-time the spaced plants were strong and healthy, well branched, bearing plenty of long well-filled pods, while the roots were very strong. In the crowded square, on the other hand, the middle plants were obviously smaller in all respects than the outer, the difference being now as noticeable in the shoots as in the roots. Most of the pods were thin and distorted, and the seeds had not developed properly.

Table I.

Dry Weights. (*Mean figures.*)

	Shoot	Root	Total	Efficiency index
Spaced plants:	3·488±·081	·474±·016	3·962±·095	2·253±·024
Crowded plants:				
Outer rank	2·478±·057	·332±·009	2·810±·066	1·953±·022
2nd	1·726±·093	·236±·011	1·962±·102	1·609±·046
3rd	1·348±·055	·173±·010	1·521±·065	1·402±·041
Inner	1·574±·061	·207±·012	1·781±·072	1·556±·039

The above table shows how seriously the reduction of light due to overcrowding affected the growth of peas. A large reduction in dry weight and efficiency index occurred at the outer edge of the square, although one side of each plant was free from light competition, and this reduction was intensified inside the square, where shading came on all sides. Apart from the outer row the differences between the plants were not very marked, showing how effective is the shading of pea plants by their neighbours when in close proximity. Broadly speaking, these results are comparable with those obtained for barley, and indicate a similar reaction of broad- and narrow-leaved plants to light deficiency. The percentage of nitrogen in the spaced plants was lower than in the crowded ones, being only 3·62 per cent. against 4·15 per cent. As with barley this probably shows that peas utilise less nitrogen in the production of each unit of dry matter when adequate illumination is available.

When, however, the above experiment was carried on under conditions of very high temperature and prolonged intense sunshine, certain

differences in behaviour manifested themselves which demanded closer investigation.

Between May 7th and June 25th, 1920, the following conditions prevailed:

Average weekly maximum temperature	27–35° C.
„ „ minimum „	8–13° C.
Total hours of sunshine (per week) ...	49·3–65·7
Daily average hours of sunshine ...	8·4

It was soon evident that the crowded plants were making the better growth; they were larger and greener than the spaced plants, some of the latter becoming yellowish, with leaves that inclined to shrivel. The crowded peas maintained their apparent lead, and when cut were mostly healthy and green, with only four casualties, whereas many of the spaced plants were pale in colour and 15 out of the 64 had succumbed.

The effect of competition was evident in the crowded square, as the outer plants averaged 2·127 ± ·065 gm. and the average of the inner ranks varied from 1·523 ± ·185 to 1·686 ± ·058. The spaced plants, however, failed to demonstrate the advantage of the extra light they had received, as their mean weight was only 1·912 ± ·042 gm., less than the outer rank of the crowded set.

A marked difference was noticeable between the plants in the spaced set. Those which were green and healthy had good stiff roots studded with rather outstanding sturdy laterals, whereas in those in which the upper leaves were turning pale the roots looked unhealthy and brown, and were flabby and inclined to be slimy. The worse the shoot the worse the root. The green healthy plants were of the normal type, with one tall shoot and large dark green leaves, whereas those with pale shoots were bushy at the base, owing to the development of axillary buds. This was apparently due to an effort to overcome some detrimental factor acting upon the spaced peas and preventing them from developing normally, for in the earlier experiments with barley the crowded plants also appeared to make the larger growth on the whole, but were not so heavy as the spaced plants when cut. Even the outer rank of the crowded plants showed the influence of this adverse factor to some extent, as the mean weight was not very much above that of the inner plants which were under the influence of more light competition. As in the first experiment described the plants within the square were all very similar in growth and weight.

The harmful effect was obviously due to the prevailing high temperatures or the excessive power of the sun's rays, or both, but the relative

importance of these two factors was by no means clear. For several years it has been noticed that plants fail to do well in the greenhouse in hot summer weather, whereas the same species flourish outdoors at the same time, and it was suspected that the high temperatures reached by the culture solutions had some connection with this phenomenon (4). An examination into temperature conditions was therefore undertaken.

In the last experiment described (p. 199), temperature readings of the nutrient solutions taken on various occasions on hot days showed very considerable differences according to the situation of the plants. Two typical records were as follows:

	Air temperature (shade)	Crowded plants	Spaced plants
June 7th, 2.30 p.m.	23° C.	Outer rank 19° C.	25·5° C.
		2nd „ 16° C.	
		3rd „ 15·5° C.	
		Inner „ 15·5° C.	
June 25th, 10 a.m.	24·5° C.	Outer rank 20° C.	22·5° C.
		2nd „ 18·5° C.	
		3rd „ 18·5° C.	
		Inner „ 18·5° C.	

On hot sunny days, therefore, the spaced plants were liable to be subjected to very high temperatures at the root, on occasion exceeding that of the air. In the crowded square, however, the outer ranks received a partial shelter from their neighbours and the solutions never became so hot, while within the square all the temperatures were usually very even, within a very few degrees, and were somewhat lower than the others. Under these conditions the crowding apparently served as a measure of protection either by keeping down the root temperature or by the reduction it effected in the amount of sunlight reaching the leaves. It is obvious that beyond a certain limit the effect of high root temperatures and of abundant sunlight became directly harmful and inhibited growth, but the extent to which each factor was responsible was not shown by the data obtained.

Further knowledge on this point was gained from a similar experiment carried on in the abnormally hot autumn of 1921, when readings were made of the daily maximum and minimum temperatures of the solutions of specified plants. No shading was applied to the greenhouse, and the sun's rays struck through the sloping roof directly on to the crowded square and some of the spaced plants, while the rest of the latter were on a side bench under a higher roof at a different angle, from which the concentration of the sun's rays seemed to be considerably less,

though the light intensity was apparently not affected. The environmental conditions were as follows:

Average weekly maximum temperature of house	23·6–31° C.
,, ,, minimum ,, ,,	10·0–15·3° C.
Total hours of sunshine (per week)	21–52
Daily average hours of sunshine	5·8

In this case the crowded plants showed less difference among themselves than usual, the outer ones averaging 1·681 gm. against 1·339 gm. for the inner. The spaced plants alongside were seriously harmed, and only produced 1·055 gm. dry matter.

The temperature records of the solutions were:

	Highest max.	Lowest max.	Mean max.	Highest min.	Lowest min.	Mean min.	Diff. between daily max. and min.
A. Spaced (under sloping roof)	29·5° C.	16° C.	26° C.	17° C.	8° C.	13·5° C.	21–2° C.
B. Corner of square	29·5° C.	15° C.	22° C.	17° C.	8·5° C.	13·5° C.	15·5–1·5° C.
C. Middle of square	23·5° C.	14° C.	18·5° C.	20° C.	12° C.	16° C.	6·5–0·5° C.

In these spaced plants the mean maximum temperature was very high, and for a period of seven successive days the solutions ran up to above 29·7° C., the highest reading of the thermometer. The differences between the day and night readings were therefore often large, although the mean minimum did not fall below that of the outer crowded square. A surprising difference was evident with the spaced plants on the side bench. These grew well and strongly, looked better than any of the crowded plants, and when cut averaged 2·171 gm. dry matter against the 1·055 gm. of the spaced set under the more sloping roof, *i.e.* they were twice as heavy. Daily temperature records were not taken for this set, but on several occasions readings were made of all the solutions, and they were always approximately the same for both sets of spaced plants.

	Oct. 5th, 2 p.m.	Oct. 6th, 2 p.m.	Oct. 11th 12 noon
Spaced, on side bench	31° C.	28·5° C.	24° C.
Spaced, under sloping roof	31° C.	28·5° C.	25° C.
Air temperature	28·5° C.	28° C.	26·5° C.

The relation of temperature to growth has been considered by various workers, but generally in connection with the rate of growth of the roots of seedlings during short periods covering a few hours at most. Under these conditions Leitch (7) found that for peas 30° C. is a critical temperature above which growth is adversely affected, 28–30° C. being the optimum, considered as the highest temperature at which no time factor is operating. Lehenbauer (6), working with maize in water cultures, showed that the optimum temperature varied with the period of

exposure, and that with prolonged exposure to the initial optimum the rate of growth falls off rapidly. It may therefore be concluded that the higher temperatures near the optimum for short exposures exercise an adverse influence when they continue to act throughout the life of the plant. Balls(2) attributed the decrease and ultimate cessation of growth at high temperatures to the accumulation of katabolic products in the cells, prolonged exposures to submaximal temperatures favouring the rapid production of these substances.

In the experiments under consideration the initial optimum of 30° C. for peas was exceeded on nine occasions, most within a single week, the highest maximum reaching 34° C. These air temperatures were only maintained for a short time, at the hottest time of the day, the period of exposure being thus very short and rare in occurrence. The average maximum temperature ruled several degrees lower, except for the one week. Furthermore the diurnal fall to the minimum temperature was considerable, 10–15° C. or more, and, as Askenasy(1) and Leitch(7) have both demonstrated that the rate of growth follows immediately and accurately any considerable change of temperature, the slowing off of growth would permit of the reduction of accumulated katabolic products and mitigate the effects of exposure to high temperatures. It would seem, therefore, unlikely that the temperatures, *per se*, were high enough to be harmful to growth, as almost the whole of the air temperature curve fell below 31° C., the initial optimum for short period exposures, especially as the root temperatures during the same period were on the whole rather lower, though they followed the air temperatures fairly closely. The adverse factor is to be sought in the intensity of the sun's rays —much depression of growth occurring where they were focussed on the leaves under the sloping roof. The different angle of incidence of the rays on to the side bench prevented such undue concentration on the leaves, and growth was correspondingly better under similar temperature conditions. This is further corroborated by comparison with the May–June (1920) experiment. In both cases the mean weekly temperatures were very similar, as the higher summer maxima were almost compensated for by higher autumn minima. The May–June plants received far more sunshine—411 hours against 262 hours, but showed less signs of distress throughout their growth, and produced 1·913 gm. dry matter as against 1·055 gm.[1] In the summer, however, the greenhouse was shaded and the

[1] For fair comparison only those plants growing in the same situation under the sloping roof are here taken into consideration though it happens that for May–June the mean of these is the same as that of the whole series (p. 199).

leaves did not receive the full force of the sun's rays, the harmful action of excessive insolation being thus mitigated, enabling the plants to make better growth than when they were exposed to the full power of the sun, although acting over a much shorter total period in the latter case. It would appear, therefore, that a high degree of insolation (excessive power of the sun's rays) is a more potent factor for harm than either high temperature or the actual total duration of sunshine.

Further experiments were undertaken to ascertain whether the harmful effects of excessive insolation could be reduced by alteration in temperature conditions. As has been already indicated, the difference between the day and night temperatures of water culture solutions is often considerable, especially in hot weather, when it may be 22·5° C. on occasion. This is considerably greater than the fluctuation occurring under soil conditions in the open, where the minimum soil temperature remains considerably above the air minimum, especially in the summer (5), and the maximum does not rise so high as in the water culture solution under glass. In dull weather the maximum and minimum temperatures approximate more closely, as there is less heating up during the day and a less marked fall in the temperature of the glasshouse at night. A method was therefore devised whereby the plants were subjected to a more even temperature at the roots, in order to ascertain whether this affected growth to any appreciable extent at different seasons of the year. The whole of the practical work in connection with this experiment was carried through by Professor Kharak Singh, of Lyallpur, India.

Two 100 gallon tanks were set up, with an outlet pipe from below the rim running down inside to within an inch of the bottom of the tank. Water was admitted from above at the other end of the tank and kept running day and night, so that a continuous slow circulation was maintained. A platform weighted with bricks to carry the water culture bottles was so arranged as to bring the necks to the rim of the tank, just above the constant level of the water. To exclude the light from the roots black cotton covers were fastened round each bottle, as the ordinary paper coats are useless when submerged, and the necks were painted with black enamel in addition. A platform of similar height and size was placed close by to carry a set of bottles in which the variation of temperature was not controlled by a water jacket, both tanks and table being under the sloping roof of the glasshouse. Under these conditions the shoots of the peas were subjected to similar insolation and air temperature, but the temperature at the roots varied with the situation. Twenty-four plants were grown in each case, spaced far enough apart

to avoid any overshadowing. Maximum and minimum thermometers were placed in several of the bottles and readings taken daily, and the nutrient solutions were changed frequently. Two experiments were carried through:

(1) In spring, during the most favourable period for growth under greenhouse conditions;

(2) In summer, during the time that premature death of the plants usually occurs.

(1) Spring Experiment.

Sutton's Harbinger Peas—April 18th to June 16th.

Growth proceeded satisfactorily with all the plants, and for some time little difference was manifest; eventually the plants on the table began to draw slightly ahead of those in the tanks, and they came into flower somewhat earlier. When cut most of the plants showed incipient signs of dying, as the upper leaves were turning yellow, indicating completion of growth, but comparatively little difference was noticeable between the two sets. The mean dry weights proved to be

	Shoot gm.	Root gm.	Total gm.	Ratio shoot/root
On table	4·284±·109	·885±·028	5·169±·132	5·121±·168
On tank	3·808±·055	·753±·020	4·561±·056	5·215±·143

The mean weekly temperatures (Fig. 1) show a difference of about 8–11° C. between the maximum of table and tank, and 3–5·5° C. between the minima. In all cases the tank maxima were below those of the table, and the minima above, as the surrounding water prevented extreme fluctuations in either direction. On the table the mean maxima ranged 15·5–22° C. above the minima, whereas in the tank the difference was only 3–5° C. Nevertheless, in spite of these considerable differences in root temperature, both as regards the actual temperature reached and the daily fluctuations between maximum and minimum, the growth of the plants was much less affected than might have been anticipated, those on the table being somewhat the heavier. The improvement in the latter case may be attributable to the higher average mean temperatures prevailing throughout the experiment, while it was also probably influenced by the rather low temperatures at the beginning, when the warmer conditions on the table gave the plants the advantage of a better start by enabling them to grow more rapidly at first. This early start was very important, as by the working of the compound interest law it gave these plants a lead which those in the tank were never able to overtake. The ratio of shoot to root was the same in each set, within experimental

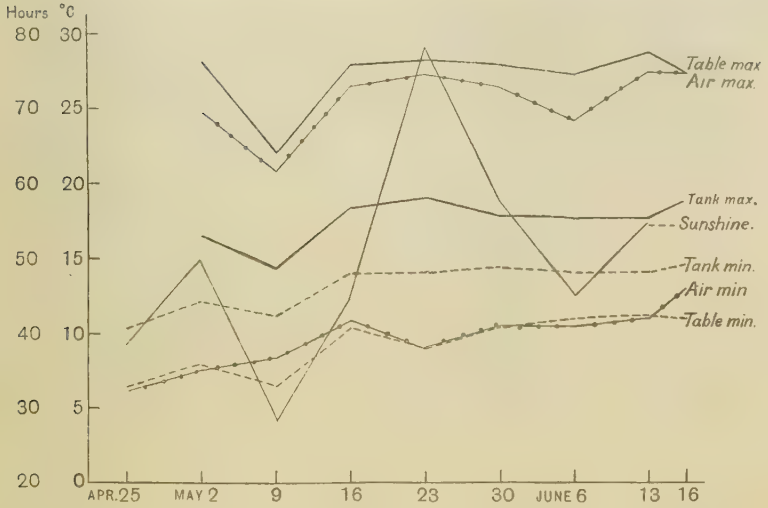

Fig. 1. Temperature records and hours of sunshine, April 19th–June 16th, 1921.

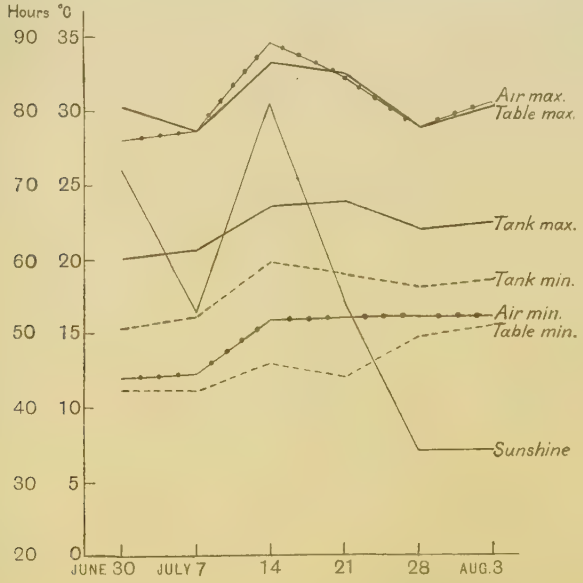

Fig. 2. Temperature records and hours of sunshine, June 24th–Aug. 3rd, 1921.

error, showing that the variable temperature had not caused any change in the development of the roots compared with that of the shoots.

The daily average of sunshine over the whole period was seven hours. During the first month the total hours per week were somewhat low, but May 16th–23rd was a very sunny week, ten to fourteen hours being registered on each of five days. After this no further period of excessive sunshine was recorded. At first the temperatures fluctuated to some degree with the amount of sunshine, but later were independent of it, for when the total sunshine dropped during the last five weeks, the mean temperature remained very constant and high, 27–28·5° C.

It would thus appear that under similar conditions of light and provided no inhibiting factor such as excessive insolation comes into play, the amount of daily fluctuation of root temperature has comparatively little effect on the growth of peas within a total mean range of 7–29° C., provided that the mean temperatures do not vary considerably. These are the limits in the experiment under consideration and possibly might be extended to some degree in either direction. Within those limits a large variation in maxima, up to 11° C., will permit of much the same amount of growth as measured by dry weight, though a low mean maximum (below 16° C.) in the early stages may cause some retardation. Growth proceeds equally well whether the temperatures at the roots are fairly even, varying within 5° C., or whether they fluctuate as much as 22° C., on the average, *i.e.* within certain limits high maximum temperatures associated with low minima have the same ultimate effect on growth as low maxima and high minima.

(2) Summer Experiment.

Sutton's Harbinger Peas—June 24th to August 3rd.

The experiment was begun in hot sunny weather when temperatures ruled very high and the number of hours of sunshine was excessive. Very soon many of the unprotected plants on the table began to show signs of distress, turning pale and wilting, and within eighteen days many were dead. In six weeks there were only four survivors, and these were small and distinctly unhappy. The plants in the tank grew well from the beginning and remained green and healthy to the end, only one failing. At the time of cutting the upper leaves were just beginning to turn yellow, showing growth was finished. The mean dry weights were:

	Shoot gm.	Root gm.	Total gm.	Ratio shoot/root
Table (4 plants only)	1·157 ± ·091	·214 ± ·026	1·371 ± ·109	5·63 ± ·478
Tank (23 plants)	1·839 ± ·007	·227 ± ·075	2·066 ± ·078	8·26 ± ·289

The plants in which the roots were protected from excessively high temperatures made therefore about half as much growth again as the unprotected survivors on the table. The increase was chiefly due to shoot growth as the roots weighed much the same in both cases, thus suggesting that the injurious action of combined strong insolation and high temperature is more marked on the assimilatory tissues than on the roots, the organs of absorption, the ratio of shoot to root being thus reduced. This is in contrast to what happens when growth is adversely affected by overcrowding, in which case the shoot/root ratio increases (3), probably owing to an attempt on the part of the plant to increase its assimilatory surface in view of the decreased illumination.

Throughout the period the mean temperatures in the solutions were from 4–5·5° C. higher than during the earlier test, all being above the highest means previously registered, but the differences between the table and tank maxima and minima were very much the same in both cases. The table maxima, however, ruled very high, ranging from 28·6–33·3° C., *i.e.* at temperatures above the initial optimum, which would cause a depression in the rate of growth during their period of operation. Added to this, there was a great deal of strong sunshine during the first and third weeks, and this association of excessive insolation with high root temperatures wrought havoc among the plants on the table, and gave them a very bad start. During the last three weeks there was a great drop in the amount of sunshine, but the temperatures remained high, so that at the end of the period the temperature effect was the more marked. The same amount of sunshine, however, had far less detrimental effect when the roots were kept cooler, and not only did nearly all the plants in the tanks survive, but they made much greater individual growth.

Nevertheless, a comparison of the dry weights shows that the conditions in the later test were less favourable even in the tank, though the depreciation was not nearly so great as on the table.

Total dry weights.

	April—June gm.	July—Aug. gm.
Tank	4·561	2·066
Table	5·169	1·371

Growth in the second experiment was practically finished in six weeks instead of in eight, but with the speeding up less than half as much dry weight was produced. This may possibly be attributed to the excessive

insolation rather than to the high root temperatures, as the tank maxima were much lower than the table maxima of the spring experiment and so were under the limits at which growth is adversely affected. On the other hand, the mean minima ranged several degrees (4–8·5° C.) higher than in the earlier test. Previous experiments with peas (3) have shown that with high maximum temperatures a rise in minima is disadvantageous and checks growth considerably. Temperatures of 13–15·5° C. are distinctly harmful when associated with 26·5–35° C. as maxima. In the present case the mean minima were higher and ranged from 15·5–20° C., being above 18·5° C. for most of the time, and may have exercised a harmful effect even though the associated mean maxima only reached 20–24° C. The total growth in the tank in summer may therefore have been depressed by the high minimum temperatures as well as by the excessive insolation, but the influence of these two factors cannot yet be dissociated.

Summary.

1. Under ordinary environmental conditions of temperature and sunlight the growth of peas, as of barley, is seriously hindered by overcrowding, even when each plant receives a similar supply of food and water. Not only is less dry weight produced, but the pods become thin and distorted and fail to develop their seeds properly.

2. Growth tends to be depressed in hot sunny weather when no protection is afforded. The chief detrimental factors concerned appear to be high temperatures at the roots associated with strong and prolonged sunshine, though the two factors acting individually are much less potent for harm. Under these conditions crowding shelters the roots from overheating and the leaves from too much sunlight, and up to a certain point crowded plants make better growth than those spaced well apart. Overcrowding, however, still depresses growth, probably because the light and root temperature reductions are too great.

3. Provided insolation is not excessive the amount of daily fluctuation of root temperature over a total range of about 22° C. (6·7–28·9° C.) has comparatively little influence upon growth; high maxima and low minima give similar results to low maxima and relatively high minima, provided the average mean temperatures are not too dissimilar.

4. With high root temperatures a difference in the degree of insolation or in the angle of incidence of the sun's rays may have a considerable influence on growth, a slight easing off of the solar conditions enabling much better growth to be made.

5. With very strong sunshine reduction of high maximum root temperatures (from 29° C. upwards) allows of satisfactory growth, when unprotected plants are rapidly killed. The inhibitory action of too high temperatures at the roots is thus clearly shown.

Nevertheless, the growth so made is less good than under more normal conditions of insolation, thus demonstrating the harmful action of too powerful sunlight, when all the root temperatures rule high.

6. Root temperatures appear to be of greater importance than atmospheric temperatures, as good growth can be made in hot atmospheres provided the roots are kept relatively cool.

7. There is some reason to believe that the minima are of as much importance as the maxima, *i.e.* that plants can withstand very high maximum temperatures provided there is a considerable drop to the minima, but cannot put up with the constant conditions of heat induced by fairly high maxima and high minima.

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STUDIES IN BACTERIOSIS. VII

COMPARISON OF THE "STRIPE DISEASE" WITH THE "GRAND RAPIDS DISEASE" OF TOMATO

By SYDNEY G. PAINE and MARGARET S. LACEY.

(*From the Department of Plant Physiology and Pathology, Imperial College of Science and Technology, London.*)

In the course of an investigation of the "Stripe Disease" of tomatoes a yellow organism was frequently found associated with the causal organism. The properties of this organism were so similar to those of *Aplanobacter michiganense*, E.F.S. (1) as to suggest close relationship with it, and in a former communication (2) the question was raised whether on further investigation the Grand Rapids Disease might not prove to have a common etiology with the "Stripe Disease."

By the courtesy of Dr E. F. Smith and Professor Nakata, to both of whom the authors wish to express their thanks, a tomato plant infected with the Grand Rapids Disease was obtained and a careful comparison of the two organisms rendered possible. From this plant *Aplanobacter michiganense* was isolated without difficulty and compared with the yellow organism referred to above. The two organisms were given preliminary culture by several transfers in broth tubes and their characteristics on different media were then investigated with the following result.

	Aplanobacter (Paine and Bewley)	*Aplanobacter michiganense* E.F.S.
Pathogenicity	Not pathogenic for tomato when associated with *B. lathyri*	Pathogenic for tomato
Bouillon-agar plate culture	Colonies round, smooth, glistening, viscous, deep orange	Colonies round, smooth, glistening, very pale yellow at first deepening with age to a mid-chrome
Bouillon-agar slope ...	Deep orange, very viscous	Pale yellow, not nearly so viscous
Broth	Slow growth, clear after 24 hours, cloudy after 48 hours, no pellicle	The same
Gelatine slope	Liquefaction starts in 48 hours	No liquefaction until the 11th day
Nitrate broth	No reduction of nitrate	The same

	Aplanobacter (Paine and Bewley)	*Aplanobacter michiganense* E.F.S.
Glucose broth	Slight acid on 6th day litmus reduced, pellicle	Slight acid on 13th day litmus not reduced, no pellicle
Lactose broth	Slight acid on 9th day litmus reduced, pellicle	Slight acid on 13th day litmus not reduced, no pellicle
Sucrose broth	Slight acid on 6th day litmus reduced, pellicle	Slight acid on 13th day litmus not reduced, no pellicle
Potato plug	Thick viscous growth, wet shining, deep orange, potato dark grey	Growth not nearly so viscous, pale yellow, potato pinkish
Diastatic action	Strong	Strong
Milk tubes	No clotting, digestion of casein apparent after 9 days, complete in 3 weeks	No clotting, casein digestion not apparent until the 14th day, only slight after 30 days
Indol formation	Slight sign	No sign
Staining	Gram positive when first isolated but later became gram negative	Gram positive
Size and shape	Small oval rods, $1{\cdot}6\mu \times 0{\cdot}6\mu$	Small oval rods, $1{\cdot}6\mu \times 0{\cdot}6\mu$
Motility	Non-motile	Non-motile

From the above it is seen that while these two organisms possess many properties in common, certain differences, mainly differences in degree only, clearly mark them as different species and the name *Aplanobacter dissimulans* is now proposed for the species isolated by Paine and Bewley.

Infection Experiments.

Inoculations of young tomato plants were carried out in the experimental houses at Waltham Cross by Dr W. F. Bewley, to whom the authors tender their best thanks. The two *Aplanobacters* and *Bacillus lathyri* were pricked separately into three sets of eighteen plants; the results with *Aplanobacter dissimulans* were negative in every case; many successful infections were obtained with *B. lathyri* and *A. michiganense*, the effect upon the pith was identical and altogether indistinguishable, but marked differences were observed when the organisms, having passed through the cortex, produced lesions on the exterior of the stem. The former produced dark brown sunken furrows with usually no cracking of the epidermis, while the latter gave no special colouring but produced deep fissures in the outer cortex whose margins had the appearance of callus formations; no effect upon the fruits was observed in the case of *Aplanobacter michiganense*. The two diseases therefore appear to be entirely distinct, and the senior author desires to withdraw the suggestion

made in the former paper, to which reference has been made above, that in his investigation of the Grand Rapids Disease Dr Smith had possibly been in error as to the etiology of the disease.

SUMMARY.

1. The Stripe Disease and the Grand Rapids Disease of tomato are distinct diseases caused by two bacterial parasites, *Bacillus lathyri* and *Aplanobacter michiganense*.

2. The yellow organism, *Aplanobacter dissimulans* n.sp. (Paine and Bewley), which is frequently found associated with *Bacillus lathyri* is not identical with *Aplanobacter michiganense*.

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THE INFESTATION OF FUNGUS CULTURES BY MITES[1]

(ITS NATURE AND CONTROL TOGETHER WITH SOME REMARKS ON THE TOXIC PROPERTIES OF PYRIDINE)

By SIBYL T. JEWSON, M.Sc.

(*Department of Mycology, Rothamsted Experimental Station, Harpenden*),

and F. TATTERSFIELD, B.Sc., F.I.C.

(*Rothamsted Experimental Station*).

(With 4 Text-figures.)

CONTENTS.

1. Introduction.

The infestation of pure cultures of fungi by mites is a considerable source of trouble in mycological laboratories. This difficulty having arisen at Rothamsted, it was considered advisable to make a careful study of the nature of the infestation and the toxic effect of a number of volatile organic chemical compounds on these pests and on fungi. The object of the investigation was to find a method of controlling the mite infection without injuring the fungi. Once having gained access to the laboratory

[1] A grant in aid of publication has been received for this communication.

mites make their way through cotton-wool plugs of culture tubes. Besides destroying the culture they have entered, they may make accurate subculturing a matter of difficulty by reason of the extraneous matter —bacteria, fungus spores, etc.—they carry with them into the tube. They wander rapidly from tube to tube and, unless discovered at an early stage, the whole set of cultures in a laboratory may be either destroyed or seriously contaminated. Even if the cultures be abandoned and a completely fresh start made, another infestation may readily take place from eggs laid in some unnoticed corner of the laboratory.

2. Nature of the Infestation.

Three species of mites were found contaminating cultures of which *Aleurobius farinae*, De Geer, the Flour Mite, was the most abundant and widespread. In many cases infection was slight; in others eggs, larvae and adults were present but the mycelium was not noticeably destroyed by this species. *Tyroglyphus longior*, Gervais, one of the cheese mites, was observed in a few cultures. In most cases infection by this species was very slight, but in three cultures of a species of the fungus *Trichoderma* the whole of the fungus was destroyed and the medium was blackened with faecal pellets. *Glyciphagus cadaverum*, Schrank, was found only in one set of cultures. The eggs of the two latter species were not recorded. These three species are among those termed "Forage Mites" as distinguished from "Mange Mites." They infect many kinds of grain and flour and can frequently be found in the dust from crevices in houses or stables. *T. longior* and *A. farinae* are also two of the species that attack Stilton and Cheddar cheeses. The life histories of all three species are very similar, consisting of four stages, egg, larva, nymph and adult. That of *T. longior* has been described by Eales(1). The life cycle is completed in four to five weeks, the eggs hatching about 10–12 days after being laid. The larva is distinguished from the later stages by having only three pairs of legs. It feeds actively for about a week, then becomes quiescent and casts its skin, emerging as the first nymph. This moults and becomes the second nymph which after a third moult emerges as an adult male or female. There may, under favourable conditions, be an additional hypopial stage, the hypopus being specially adapted for distribution. It has a resistant skin and on the ventral surface there is a sucker by which it can attach itself to flies, moths or human beings. The life cycle of *A. farinae* as described by Newstead and Duvall(2) is very similar but usually shorter, varying from about 17 days in July to 28 days in the winter months. The eggs usually hatch in about 3–4 days. There is only one nymphal stage and the hypopus is very rare. *G. cadaverum* has a similar life history but the details are not well known.

3. THE PROBLEM OF CONTROL.

The *sine qua non* of any method of control is that the treatment should kill 100 per cent. of the mites and their eggs and have a minimum detrimental effect upon the fungus cultures. It should not be harmful to the operator and it should be easy to apply. If a chemical method is to be used it is essential that the substance be volatile, not too disagreeable, and that in its toxic action it should be reasonably speedy. In flour mills it is customary to keep mites under control by scrupulous cleanliness and where necessary by the application of heat. The lowest lethal temperature was found by Newstead and Duvall(2) to be 49° C. applied for at least 12 hours. This latter method was not available in our case as the temperatures likely to be effective against the parasite would have a seriously detrimental action upon the fungus culture. A fairly extensive list of volatile organic compounds was therefore tried and their effect studied upon mites and their eggs and upon fungi.

Ammonia was found to be the most rapidly toxic substance to mites and their eggs. It had, however, a definite toxic action on fungi and although it may prove of great value for ridding laboratory apparatus, such as incubators, of these pests, its vapour should not be allowed to play upon the cultures of fungi for any length of time.

Pyridine was the next most rapidly toxic compound tested and although it is many times less toxic than ammonia vapour, it has the added advantage of not being poisonous to fungi, except in doses not likely to occur in practice. As its vapour is rather disagreeable it is hardly suitable for the purposes for which ammonia is recommended, but for freeing fungus cultures of mite pests it can be so easily applied that it should not prove in any way obnoxious to the operator. A detailed description of both methods is given on p. 239.

4. EXPERIMENTAL.

The compounds tested were:

Ammonia bases	Ammonia
	Pyridine
	Aniline
	Monomethylaniline
	Dimethylaniline
Aromatic hydrocarbons	Benzene
	Toluene
	Naphthalene
	Para-dichlorbenzene
	Carbon tetrachloride
	Carbon bisulphide

All these compounds were chosen because of their definite insecticidal value. With the exception of Ammonia, Mono- and Dimethylaniline this is not considerable but it was thought to be sufficiently high for the substances to prove effective in air saturated with their vapour against a not very resistant pest. Moreover, it was considered that the toxic effect of the majority of them to fungi would be small.

(*a*) *Action upon Fungi.*

As it was essential that the latter condition should be complied with, these substances were all given a preliminary test to discover their action against a common fungus. A green *Penicillium* was used, the cultures being tested in duplicate, one of each couple being exposed with the cotton-wool plug *in situ*, the other with it removed[1]. The culture tubes were put into a large boiling tube containing a quantity of the chemical, sufficient to saturate the air with its vapour. The boiling tube was then corked and put aside for three days after which the culture was taken out and subcultured. The results are stated in Table I.

Table I.

Effect of Vapour of various Organic Chemicals on Penicillium *sp. Culture exposed for three days and then subcultured.*

	Chemical	Growth of subculture after 7 days
1.	Ammonia	No growth
2.	Pyridine	Good growth
3.	Aniline	,,
4.	Monomethylaniline	,,
5.	Dimethylaniline	,,
6.	Benzene	,,
7.	Toluene	Fairly good growth
8.	Naphthalene	Good growth
9.	*p*-Dichlorbenzene	Good growth
10.	Carbon tetrachloride	One fair growth and one slight growth
11.	Carbon bisulphide	No growth

(*b*) *Action upon Mites.*

A selection of the above compounds was then tested upon mites. Some flour mites, *A. farinae*, were placed in tubes which were vaselined round the outer lip to prevent the escape of the mites, but left unplugged. Exposure to the toxic substance was made in exactly the same way as described but for varying lengths of time. The results are shown in Table II.

[1] The results of the two series showed no significant differences.

Table II.

Action of the Vapour of Certain Organic Chemical Compounds on Mites (Aleurobius farinae).

Chemical	Action after 3–4 hours	Action after 16 hours
Pyridine (1) Pure ...	—	All appeared dead.
„ (2) ...	After 4 hours all mites appeared dead. Some of larger ones recovered a day later and some eggs hatched out.	—
„ (3) Commer'l.		All appeared dead.
Aniline		Many appeared dead but most of the large and some small ones moved sluggishly.
Monomethylaniline	–	A few of both large and small ones alive but sluggish.
Dimethylaniline	—	Large mites alive but sluggish.
Naphthalene ...	No apparent effect.	No apparent effect.
p-Dichlorbenzene ...	All anaesthetised.	All apparently anaesthetised but some recovered on exposure to air.
Carbon bisulphide	All apparently dead.	All apparently dead.

An inspection of Tables I and II clearly indicates that for practical purposes Pyridine is much the most hopeful compound. Carbon bisulphide, although apparently rapid in its action, is too toxic to fungi to be useful, while Paradichlorbenzene, which, from its slight poisonous action on fungi and from the almost complete absence of disagreeable properties, would have been an ideal substance to apply, seems to have only a pronounced but temporary anaesthetic effect.

In view of these results it was decided to make a more complete study of the toxic action of Pyridine and to ascertain, if possible in a quantitative way, its reaction with both mites and some common fungus.

5. The Quality of the Pyridine used in the Experiment.

Four samples of Pyridine were tested for their effect upon mites and fungi.

1. A sample labelled pure Pyridine.
2. A sample obtained by a rough fractionation of commercial Pyridine.
3. Commercial Pyridine.
4. A sample carefully purified in the Laboratory.

No. 2 sample was intermediate in quality between samples 1 and 3.

The testing of these grades was regarded as necessary owing to the wide discrepancy in price between pure and commercial Pyridine. It was also essential to ascertain whether through the presence of any

impurity commercial Pyridine would prove deleterious to fungi and so inhibit its use or render its fractionation and purification indispensable. Moreover, it was important to ascertain whether the actual toxic product in the commercial article was Pyridine itself or some impurity.

For purposes of reference and comparison the specific gravity and the fractions distilling at various temperatures were determined.

The distillations were carried out in the following way:

75 c.c. were distilled at a rate of one drop per second from a 150 c.c. flask (neck 9 cm. long, diameter of bulb 6·5 cm.) fitted with a four-pear fractionating column of a length from bottom to side tube of 24·5 cm.

The column was so adjusted into the neck of the flask that the total length of still-head was just about 30 cm. A Davies double jacketed condenser in a perpendicular position was attached to the side tube of the column. The distillates were collected and measured. The results are tabulated in Table III.

Table III.

Fractional Distillation of Three Samples of Pyridine.
75 c.c. distilled at a rate of 1 drop per second.

Sample of Pyridine labelled Pure. S.G. at 15·5° C., 1·0098				Crude Pyridine fraction from Commercial Pyridine. S.G. at 15·5° C., 1·0092				Commercial Pyridine. S.G. at 15·5° C., 0·99307			
No.	Temp. ° C.	Vol. of distillate	% of distillate	No.	Temp. ° C.	Vol. of distillate	% of distillate	No.	Temp. ° C.	Vol. of distillate	% of distillate
	92	1st drop			88	1st drop			94	1st drop	
1.	92–110	0·2 c.c.	·27	1.	88–110	3·5 c.c	4·67	1 *a*.	94–100	30 c.c.	40·0
								1 *b*.	100–110	5·3	7·07
2 *a*.	110–115	1·3	1·73	2.	110–120	13·5	18·00	2 *a*.	110–115	2·0	2·67
2 *b*.	115–120	25·0	33·34					2 *b*.	115–120	4·0	5·33
3 *a*.	120–125	30·0	40·0	3 *a*.	120–125	29·5	39·33	3 *a*.	120–125	16·3	21·73
3 *b*.	125–130	8·0	10·67	3 *b*.	125–130	10·5	14·00	3 *b*.	125–130	6·9	9·2
4.	130–140	7·7	10·26	4.	130–140	9·0	12·0	4.	130–140	7·0	9·33
5.	Residue	2·8	3·73	5.	140–150	6·5	8·67	5.	Residue	3·5	4·67
					Residue	2·5	3·33				

An inspection of this table shows that there are wide differences in the composition of the three samples, that the commercial product (which contains about 34 per cent. water) has a considerable fraction distilling off between 95° and 100° C., and that the sample labelled Pure is misrepresented. The last point was confirmed by testing with Permanganate which was rapidly decolourised. In view of the obvious impurity of the Pyridine labelled "Pure," this sample was treated with Potassium Permanganate, dried over solid Caustic Potash and frac-

tionated. The fraction distilling between 115° and 125° C. was again treated with Permanganate and again fractionated. The fraction distilling between 114° and 117° C. was collected and tested quantitatively for its toxicity to mites. The pure and commercial Pyridine were tested for their toxic action to mites and their eggs.

Two samples of cheese mites were obtained and identified as *T. longior*, or a species very closely related to it. Both samples contained a large number of eggs. Duplicate tubes of the mites were then treated in bell-jars with (*a*) Pyridine (1 above), and (*b*) commercial Pyridine (3 above) for a period of 16 hours, two controls being set aside over water for purposes of comparison. At the end of this period they were examined and in all the treated samples the mites showed no signs of life.

After a period of fourteen days all the samples were re-examined with the result that whereas one of the controls showed many young and lively mites and comparatively few unhatched eggs and the other a few large live mites and a large number of unhatched eggs, the tubes exposed to the vapour of both samples of Pyridine contained no live mites, either adults or newly hatched larvae. This and many subsequent experiments amply proved that there is little or no difference in toxic action between the costly pure Pyridine and the cheap commercial article.

6. Toxicity of Pyridine to the Eggs of Mites.

The critical point in the method is the toxicity of Pyridine to the eggs of mites, for unless all are killed the infection is not eliminated. This matter was therefore studied with considerable care, the actual experiments being repeated several times to eliminate chance results due to such factors as the Pyridine not penetrating a thick mass of mites or to the sample undergoing desiccation during the aeration subsequent to the experiment.

The results in one case (Series I) do not agree with those obtained at any other time, but they are set out in Table IV*a* with the purpose of indicating that a sixteen-hour exposure which we have generally found to be ample to kill all mites and eggs may fail in certain cases and as a consequence we suggest that with very heavy infestations a second exposure may be necessary after a period of fourteen days.

Series I was exposed in duplicate for 16 hours to vapour of three qualities of Pyridine. A little flour was placed in each tube to provide a food supply for any larvae hatching out. After treatment both mites and flour were transferred to fresh tubes and the excess of Pyridine allowed to escape. The result was definitely negative and might be due

either to the cold weather prevailing at the time diminishing the concentration of Pyridine in the air of the bell-jar, or to the eggs being rather more resistant in this case or under these conditions.

Series II was exposed to the vapour of commercial Pyridine for two different periods, 16 and 48 hours, in each case in duplicate. The samples were then transferred to Petri dishes and exposed to the moist air of a warm greenhouse for eight hours to free the material from traces of Pyridine as completely and rapidly as possible. The samples were then transferred back to tubes and allowed to stand in a damp atmosphere for 16 to 19 days, small portions being examined from time to time.

The results of both series are shown in Table IV*a*.

Table IV*a*.

Showing effect of Pyridine upon Mites and Eggs.
(Sample contained about equal numbers of each.)

Series I.

Exposure to Vapour of Pyridine for 16 *hours.*

Set on 18/1/22.

Examined	Days after taking off	Controls	Pure Pyridine (1)	Commercial Pyridine fractionated (2)	Commercial Pyridine (3)
19/1/22	—	Active—all stages	Apparently dead unhatched eggs	Apparently dead unhatched eggs	Apparently dead unhatched eggs
23/1/22	4	,, ,,	One mature live mite	Apparently dead unhatched eggs	Apparently dead unhatched eggs
27/1/22	8	,, ,,	One tube app. dead; second tube live larvae	Live larvae (both tubes)	Apparently dead unhatched eggs
31/1/22	12	,, ,,	One adult, one larva; unhatched eggs	Many larvae; unhatched eggs	One live larva; unhatched eggs
7/2/22	19	,, ,,	Eggs, nymphs and adults	Eggs, nymphs and adults	Eggs, nymphs and adults

Series II.

Exposure to Vapour of Pyridine for 16 *and* 48 *hours.*

Set on 20 and 21/2/22.

Examined	Days after taking off	Controls	Commercial Pyridine	
			Exposure 16 hrs.	Exposure 48 hrs.
22/2/22	—	Active—all stages	All apparently dead	All apparently dead
27/2/22	5	,, ,,	No live mites	No live mites
28/2/22	6	,, ,,	,, ,,	,, ,,
3/3/22	9	,, ,,	,, ,,	,, ,,
6/3/22	12	,, ,,	,, ,,	,, ,,
10/2/22	16	,, ,,	,, ,,	,, ,,

The eggs are obviously much more resistant than the mites themselves and it is apparent from the discrepancy between Series I and II that a slight change in the conditions of carrying out the experiment may lead to failure. In view of this a fresh series of experiments (III) was set up, the results being shown in Table IV*b*, which also gives the action of Ammonia vapour on the eggs.

Table IV*b*.

Series III. *Effect of Pyridine and Ammonia on Eggs of Cheese-mites.*

Temp. of exposure 18°–19° C.
Exposure in flasks sealed with lead-lined stoppers.
Air of flask saturated with vapour.
Time elapsing before examination is reckoned from time of taking out of flasks.

Pyridine.

No.	Description	Duration of exposure	Exam'd after	Mites alive	Eggs not hatched	Exam'd after	Mites alive	Eggs not hatched	Exam'd after	Mites alive	Eggs not hatched
1.	Control (*a*) (dry flask)	72 hrs.	14 days	Many	Some	18 days	Many	Some	25 days	Many	Few
2.	Control (*b*) (damp flask)	72 ,,	14 ,,	,,	Some	18 ,,	,,	Some	25 ,,	,,	,,
3.	Pyridine (pure)	3 ,,	17 ,,	,,	V. few	21 ,,	,,	V. few	28 ,,	,,	,,
4.		8 ,,	17 ,,	,,	Many	21 ,,	,,	Many	28 ,,	,,	Many
5.		16 ,,	16 ,,	None	,,	20 ,,	None	,,	27 ,,	None	,,
6.		24 ,,	16 ,,	,,	,,	20 ,,	,,	,,	27 ,,	,,	,,
7.		36 ,,	—	—	—	19 ,,	,,	,,	26 ,,	,,	,,
8.		48 ,,	—	—	—	19 ,,	,,	,,	26 ,,	,,	,,
9		72 ,,	—	—	—	18 ,,	,,	,,	25 ,,	,,	,,
10.	Pyridine (commer'l)	3 ,,	17 ,,	Many	V. few	21 ,,	Many	V. few	28 ,,	Many	Few
11.		8 ,,	17 ,,	,,	Many	21 ,,	,,	Many	28 ,,	,,	Many
12.		16 ,,	16 ,,	None	,,	20 ,,	None	,,	27 ,,	None	,,
13.		24 ,,	16 ,,	,,	,,	20 ,,	,,	,,	27 ,,	,,	,,
14.		36 ,,	—	—	—	19 ,,	,,	,,	26 ,,	,,	,,
15.		48 ,,	—	—	—	19 ,,	,,	,,	26 ,,	,,	,,
16.		72 ,,	—	—	—	18 ,,	,,	,,	25 ,,	,,	,,

Ammonia.

Series I.

No.	Description	Duration of Exposure	Examined after	Mites alive	Eggs not hatched	Examined after	Mites alive	Eggs not hatched
1.	Control	—	10 days	Many	Few	—	—	—
2.	,,	—	,,	,,	,,	—	—	—
3.	Ammonia	2 mins.	,,	,,	,,	—	—	—
4.	,,	5 ,,	,,	,,	,,	—	—	—
5.	,,	15 ,,	,,	None	Many	17 days	A few	Many
6.	,,	30 ,,	,,	A few	,,	,,	,,	,,

Series II.

1.	Control	—	20 days	Many	—	23 days	Many	—
2.	Ammonia	15 mins.	,,	A few	Many	,,	Some	Some
3.	,,	30 ,,	,,	None	,,	,,	None	,,
4.	,,	60 ,,	,,	,,	,,	,,	,,	Many
5.	,,	120 ,,	,,	,,	Some	,,	,,	,,

In Series III the lethal chambers were conical flasks of about 1200 c.c. capacity. Small samples containing many eggs and a few mites (100 to 28) were placed in small tubes, the mouths of which were covered with fine silk gauze, and suspended in the vapour in the flask at a temperature of 18°–19° C. for varying lengths of time. The flasks were hermetically sealed by lead-lined rubber stoppers. After exposure the samples were poured out into small flat dishes and exposed to the atmosphere for 15 to 30 minutes until the odour of Pyridine had almost disappeared. They were then placed in a large bell-jar containing a basin of water and left overnight, after which they were transferred to tubes with the addition of a little flour and kept at 18°–19° C. in a moist atmosphere.

Two samples of Pyridine, one practically chemically pure, the other labelled commercial, were tested in this way for times ranging from three hours, which is just long enough to eliminate the mites, to 72 hours. It is of interest to note that in every case except the controls, the samples became covered with a mat of fungus mycelium, indicating that little danger to fungus growth is to be feared from exposures up to 72 hours. Examination was carried out from time to time up to 28 days. Three hours' exposure was quite ineffective against the eggs, practically all hatching out in 16 days; 8 hours was partially successful as many eggs did not hatch in 20 days, while 16 hours and upwards completely prevented hatching out. No difference whatever could be detected in the lethal properties of the two samples of Pyridine. It is considered that if the treatment be carried out at an equable temperature of about 18°–20° C., 16–24 hours' exposure should be sufficient to eliminate both mites and eggs. It is recognised, however, that there may be cases of heavy infestation when the vapour of Pyridine may not be able to permeate completely and where a second exposure after fourteen days might be advisable before subculturing.

7. Action of Pyridine on Mites (Quantitative).

An attempt was made to put the results on a quantitative basis. This was deemed advisable because of the rather surprisingly high toxicity of Pyridine in air saturated with its vapour and because materials like Aniline and Dimethylaniline which, from the work of Tattersfield and Roberts (3), were expected by us to have a higher toxic value in the vapour phase than Pyridine had proved of doubtful value. Pyridine, Ammonia and Aniline were therefore compared. For this purpose flasks of about 1100 c.c. capacity were fitted with lead-lined rubber stoppers, through which passed a glass rod turned to a hook at the lower end, to

which could be attached a short test tube by means of wire. The first series of experiments was carried out in air saturated with the appropriate vapour. In the case of Pyridine and Aniline a few drops in excess of what was required to saturate the atmosphere were pipetted into the flasks. After a time sufficiently prolonged to allow of the air being saturated, the tubes containing the mites and closed at the top by a little silk fabric of very fine mesh, drawn tight and fastened firmly to prevent the mites from escaping, were inserted by attaching to the hooks and pushing the cork home. With Ammonia 5–10 c.c. of ·880 material was poured in; in this case the toxic action is so rapid as to render the silk fabric unnecessary. Two controls were used for each set of experiments. After varying lengths of time, in the case of Ammonia reckoned in seconds, the tubes were taken out and either examined immediately or after a time.

The method of examination and the time that should elapse before it is carried out were matters of considerable difficulty and need some consideration. It is necessary to count at least a hundred mites to obtain reliable results. Preliminary experiments showed olive oil to be the best medium in which to count the mites under the microscope as they remain alive in it for one to two hours and its clearing action is marked. Those mites which on careful examination showed no sign of movement were regarded as dead. If inspection be carried out immediately after exposure there is a possibility of mistaking temporary anaesthesia or stupefaction for death. Experience showed, however, that this difficulty was not very serious, for the poisons tested appear to act on the motor nerves and a mite once thoroughly incapacitated in this way seems rarely to recover. As a matter of fact, immediate examination gives an underestimation of the toxic action—but this can hardly be avoided. The dangers of allowing the material to stand overnight appear more serious as even when aerated in open dishes it loses such toxic materials as Pyridine and Aniline only after a little time and at different rates owing to differences in their respective vapour pressures, during which time the poison continues to act. Moreover, in this treatment there is a danger of desiccation, and of some non-poisoned eggs hatching out.

The effect of Pyridine was tested in two ways. The examination in one case was carried out immediately. In the other the treated mites were aerated in the open till the characteristic odour had disappeared; they were then kept for a further sixteen hours in a moist atmosphere, after which they were examined. In the case of Ammonia examination was carried out immediately and after the lapse of an hour or two during

Table V.

Toxicity of Ammonia (NH_4OH), Pyridine and Aniline to Mites. Atmosphere saturated to chemical.

Vapour from Ammonia ·880. Examination immediately after treatment.

Time of exposure	No. alive	No. dead	% alive	% dead	% calculated on live mites in control: % alive	% dead
Controls	90	10	90	10	100	—
A. 16 secs.	87	13	87	13	96·6	3·4
B. 32·2 „	78	32	70·9	29·1	78·7	21·3
C. 46·2 „	72	38	65·5	34·5	72·7	27·3
D. 60·5 „	172	138	55·4	44·6	61·5	38·5
E. 75 „	51	306	14·3	85·7	16	84
F. 90 „	44	384	10·2	89·8	11·3	88·7
G. 105 „	8	271	2·8	97·2	3·1	96·9
H. 120·5 „	10	263	3·6	96·4	4	96

Vapour of pure Pyridine. Examination immediately after treatment.

Control	144	19	88·3	11·7	100	—
A. 30 mins.	118	31	79·2	20·8	90	10
B. 60 „	121	45	72·9	27·1	82	18
C. 90 „	44	55	44·4	55·6	50	50
D. 120 „	20	77	20·6	79·4	23	77
E. 150 „	10	97	9·4	90·6	10	90
F. 180 „	1	130	0·77	99·23	1	99
G. 210 „	1	106	0·94	99·06	1	99
H. 240 „	0	113	0	100	0	100

Vapour from Ammonia ·880. Examination 1–2 hrs. after treatment.

Controls	44, 43	6, 7	87	13	100	0
A. 20 secs.	39, 40	11, 10	79	21	91	9
B. 40 „	26, 20	29, 35	41·8	58·2	47	53
C. 60 „	2, 0, 3, 1	48, 50, 47, 49	3	97	3	97
D. 80 „	1, 1, 0, 4	49, 49, 50, 46	3	97	3	97
E. 100 „	0, 0, 0, 0	50, 50, 50, 50	0	100	0	100

Table V (*contd.*)

Vapour of pure Pyridine. Examination after 16 hours.

Time of Exposure	No. Alive	No. Dead	% Alive	% Dead	% calculated on live mites in control: % Alive	% calculated on live mites in control: % Dead
Controls	50 40	5 5	90	10	100	0
A. 7·5 mins.	86 89	18 11	85·8	14·2	95	5
B. 15 „	132	13	91	9	100	0
C. 22·5 „	130	22	85·5	14·5	95	5
D. 30 „	81 83	32 17	77	23	85·5	14·5
E. 37·5 „	83 85	17 15	84	16	93	7
F. 45 „	45	55	45	55	50	50
G. 52·5 „	45	55	45	55	50	50
H. 60 „	3	97	3	97	3	97
I. 67·5 „	7	98	6·6	93·4	7	93
J. 75 „	6	94	6	94	7	93
K. 82·5 „	6	94	6	94	7	93
L. 90 „	0	100	0	100	0	100
Vapour of pure Aniline. Examination immediately after treatment.						
Control	86	14	86	14	100	—
A. 60 mins.	81	19	81	19	94	6
B. 120 „	43	57	43	57	50	50
C. 180 „	5	95	5	95	6	94
D. 240 „	2	98	2	98	2	98
E. 300 „	1	99	1	99	1	99

which time the vapour had escaped. An inspection of Table V and Fig. 1*a* shows that immediate examination rather understates the effect of the poison. The proportion of live to dead mites was counted in both treated tubes and controls and the percentage of survivors in the tests to the live mites in the controls calculated. This percentage was plotted against time. The results are set out in Table V and Fig. 1*a*.

Fig. 1*a* shows that air saturated with Pyridine is rather more toxic than when saturated with Aniline. This does not mean that weight for weight or molecule for molecule Pyridine is more toxic than Aniline; as the latter having a lower vapour pressure at ordinary temperatures (15°–18° C.) would saturate air with less of that material (weight for weight) in the vapour phase. The curves take the usual sigmoid form characteristic of such reactions. Henderson Smith(4) who studied the toxic action of Phenol on Botrytis spores obtained curves of a similar

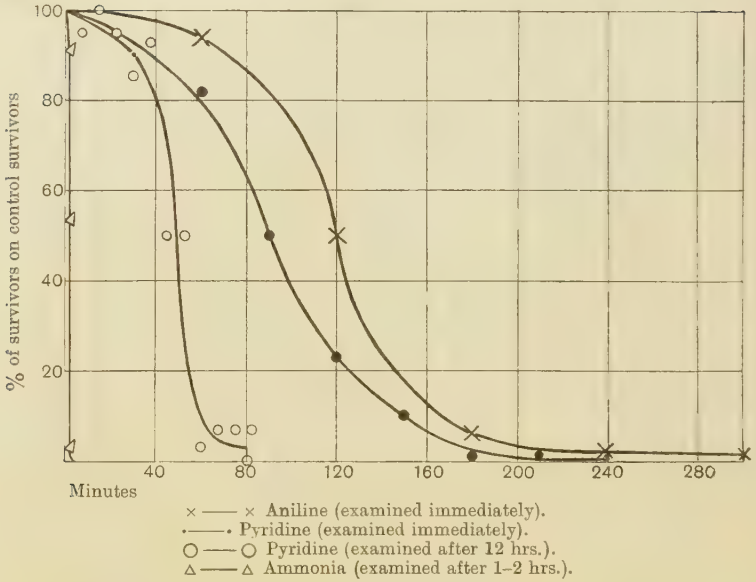

Fig. 1 *a*. Toxicity of vapours of Ammonia, Pyridine and Aniline to mites.

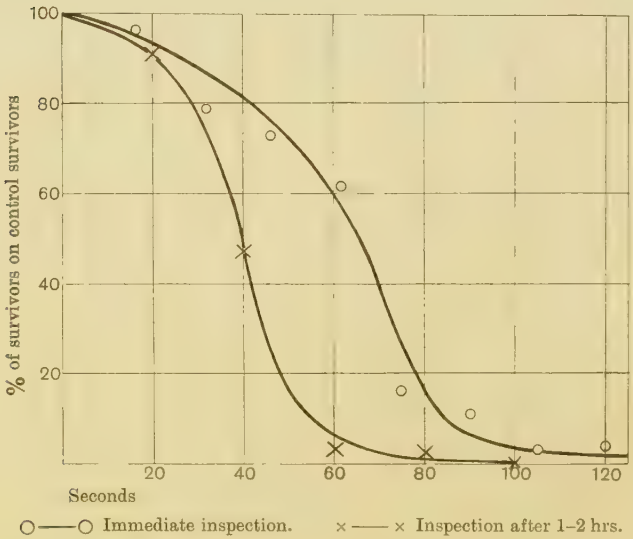

Fig. 1 *b*. Toxicity of Ammonia vapour to mites.

type, and showed that if the strength of the phenol be progressively raised the curve approximates to the logarithmic type, but that both types of curve are explicable by assuming variations of resistance amongst the spores.

In our case, working at the saturation point of poison in air, a similar method was impossible, but even with so rapidly acting a poison as Ammonia the curve obtained was distinctly sigmoid in type when survivors were plotted against time expressed in seconds instead of minutes. Curves of this type would be expected in our case, where we have in an inseparable mixture, adult mites of various ages, larvae and nymphs. The distribution of the resistances varying in all three stages of development would be complex, and the variations so great that with the most highly toxic of materials the survival curve would be of the type obtained. The toxic action of Ammonia proved so rapid that it is not expressible with accuracy on the same scale as that of Pyridine and Aniline, and is put therefore on a second scale in Fig. 1*b*.

In view of the above results it seemed of importance to ascertain whether the considerable toxic action of Pyridine could be regarded as specific. Tattersfield and Roberts(3) showed that molecule for molecule Aniline in the vapour phase was about three times as toxic to wireworms as Pyridine. For this purpose minute but progressively increasing quantities of Pyridine or of Aniline were inserted by means of a graduated capillary pipette into calibrated flasks (1100–1200 c.c.) fitted with lead-lined rubber stoppers and glass hooks, and the cork inserted. When the material had evaporated, mites (about the same number in each case) were introduced in small test tubes closed by silk gauze and attached by wire to the hook and allowed to stand for a period of three hours. This time was convenient as permitting each set of tests to be comfortably finished in one day and as giving nearly 100 per cent. of deaths with a saturated concentration of Pyridine in air. The counts were made in the usual way. The results are shown in Table VI and percentages of survivors plotted against millionths of gramme-molecular concentrations of poison in a litre of air, in Fig. 2.

The curve for Pyridine is distinctly sigmoid in character, indicating that equal increases in concentration do not have a corresponding effect. An increase of dose from 30 to 50 millionths of a gramme-molecule shows little or no increase in toxic action, but an increase of from 50–70 accounts for 75 per cent. of the mites while further increases up to near the saturation point produce effects only very gradually. The curve for Aniline is not complete, as towards the lower end of the curve the flasks are

saturated with vapour, the slowing down of the reaction being undoubtedly due to this cause.

Table VI.

Showing Toxic Effect of increasing doses of Pyridine and Aniline to Cheese-mites (T. longior).

Pyridine. Exposure 3 hours. Temp. 16°–18° C.

	Vol. of flask c.c.	c.c. added	c.c. added per 1000 c.c.	Wt. per 1000 c.c. of air	Millionths of gm. mol. per 1000 c.c. of air (approx.)	No. alive	No. dead	% alive	% alive calculated on live mites in control
Control	—	—	—	—	—	117	34	77·5	100
A.	1156	·002	·00173	·0017	21·5	75	25	75	97
B.	1191	·004	·0033	·0032	40	75	25	75	97
C.	1172	·006	·0051	·005	63	32	52	38	49
D.	1192	·008	·0067	·0066	83·5	14	91	13	17
E.	1175	·01	·0085	·0083	105	9	78	10	13
F.	1178	·012	·01	·0098	124	10	101	9	11
G.	1169	·014	·012	·0118	150	2	98	2	3
H.	1183	·016	·0135	·0132	167	3	89	3	4
I.	1158	·018	·0155	·015	190	0	90	0	0

All the mites after treatment with Pyridine extremely sluggish, and appear paralysed.

Aniline. Exposure 3 hours.

Control	—	—	—	—	—	174	62	74	100
A.	1156	·001	·00086	·00086	9	93	51	65	88
B.	1191	·002	·0017	·0017	18	58	80	42	57
C.	1172	·003	·0026	·0026	28	29	85	25·4	34
D.	1192	·004	·0033	·0033	36	32	130	20	27
E.	1175	·005	·0043	·0044	47	27	143	16	21
F.	1178	·006	·005	·005	54	22	137	14	19
L.	1167	·018	·015	·0153	164	25	112	18	25

All the live mites after Aniline treatment more active than in case of Pyridine. All flasks saturated from D downwards before treatment. C doubtful.

On these curves the 50 per cent. survivor points correspond to 21 millionths of a gramme-molecule of Aniline and 63 of Pyridine, indicating that at this point, the most suitable one for purposes of comparison, Aniline is about three times as toxic as Pyridine. Taking the Aniline curve as a whole, the break towards the end due to the fact that from about 35 millionths of a gramme-molecule upwards the air is saturated with vapour, shows that its toxic inefficiency is not due to an intrinsic lack of poisonous properties, but that its low vapour pressure limits to this extent its concentration in air at ordinary temperatures.

From a qualitative point of view Pyridine seems to have an entirely characteristic reaction. In the case of Aniline the survivors up to the 50 per cent. death point are fairly active. With Pyridine, however, from the smallest dose upwards the survivors are obviously seriously incapacitated; they appear to be suffering from motor paralysis and are only just capable of twitching mouth parts and legs. This unfortunately cannot be expressed graphically, but the condition is so marked as to indicate that Pyridine has a very powerful and possibly a specific toxic action on these pests. So small a concentration as ·0017 c.c. in 1000 c.c. of air is

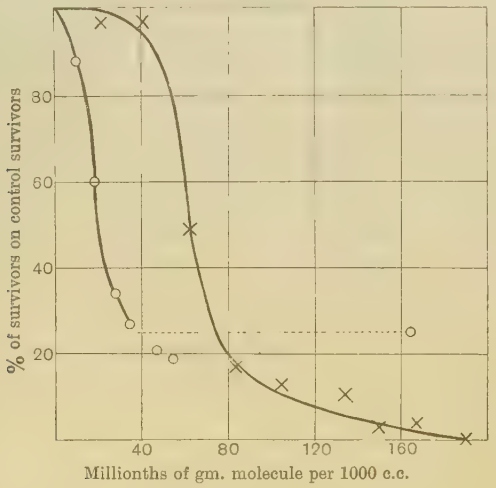

Fig. 2. Toxic effect on mites of increasing concentrations of the vapours of Pyridine and Aniline.

capable of almost completely paralysing in three hours nearly 100 per cent. of the mites.

8. Toxic Effect of Pyridine on Fungi.

After preliminary trials had shown that Pyridine was successful in eliminating mites a large number of infected cultures were treated with the vapour of Pyridine overnight (16 hours). The infection consisted chiefly of *A. farinae* with a large number of eggs, some *T. longior* while several cultures of *Mucor* were also infected with *G. spinipes* or *G. cadaverum*. The cultures were examined a week after treatment and there

was no recovery of mites or hatching out of eggs. They were then subcultured on Czapek's agar and all the subcultures grew and were apparently unaffected by the Pyridine. The six cultures of *Mucor* were, however, contaminated with *Penicillium*. A possible explanation of this seems to be that the mite *Glyciphagus*, present only in these tubes, has long hairs capable of carrying *Penicillium* spores. The cultures treated were of species isolated from the soil and were as follows:

Mucor hiemalis (Wehmer), *Botrytis pyramidalis* (Sacc.), Johnson, *Hormodendrum cladosporioides* (Fres.), Sacc., *Gliocladium penicillioides Corda* (Icon.), *Stachybotrys alternans* Bonord., *Monosporium* sp., *Fusarium*, sp. 1, *Fusarium*, sp. 2, *Penicillium*, sp. 1, *Penicillium*, sp. 2.

Nine other unidentified species including one of the Sphaeropsidales and a Dematiate form were also treated, the total number of cultures being 78. Since this experiment the method of treatment has been used a number of times and has been successful with one possible exception. In the latter case three cultures which had been treated were found some months later to be infected, but as they were among other newly infected cultures it was impossible to tell whether this was due to the failure of the original treatment or to re-infection. It was decided, in view of these results, to carry out a few quantitative experiments on the toxic effect (if any) of Pyridine to some common fungus, in order to ascertain how far this treatment could be carried with safety. The fungus chosen was *Aspergillus niger*, since work on the effect of Pyridine and various organic bases on this organism had been carried out by Brenner (5) and Lutz (6).

Into each of a series of conical flasks of 500 c.c. capacity, 200 c.c. of a suitable liquid medium was introduced, and sterilised. Gradually increasing amounts of pure Pyridine were added, and the flasks inoculated with 5 c.c. spore suspension. After a period the cultures were filtered, thoroughly washed by decantation, dried and weighed. After some preliminary experiments Coons' solution containing double the amounts of all the ingredients was decided upon as giving in a reasonable time a yield of a suitable amount for both washing and weighing purposes[1].

The most rapid and efficient filter was a Gooch crucible used with a pad of cotton-wool and under a not too high vacuum. The Gooch

[1] The medium contained in 1000 c.c

	gm.
Magnesium sulphate	0·986
Potass. bi-phosphate	2·720
Asparagin	0·530
Maltose	7·200

crucibles and fungus were then dried for 24 hours at 70° C. and finally at 90°–100° C. to constant weight.

The technique of the method has not yet been completely studied by us, but it proved of sufficient accuracy for the purpose of this investigation. Experiment No. 1 was carried out on an *Aspergillus* sp. isolated from an onion. The cultures were incubated at 26° C. for a period of seven days. Table VII shows that the growth of this fungus is not inhibited until a concentration somewhere between ·318 and ·636 per cent. of Pyridine is reached.

Table VII.

Effect of Pyridine on Aspergillus niger.

Culture solution Coons' double strength 200 c.c. Temp. 26° C.

	Description	Remarks after 3 days	Crop yield after 7 days
1.	·159 % = ·002 gm. mol. Pyridine per 100 c.c.	Growth equal to control	·3914 gm.
2.	·318 % = ·004 „ „ „	Reduced growth; small colonies	·4044 „
3.	·636 % = ·008 „ „ „	No growth	No growth
4.	1·272% = ·016 „ „ „	No growth	No growth
5.	Control	Good growth	·3536 gm.
6.	„	„	·3845 „
7.	„	„	·3942 „
8.	„	„	·3620 „

It was desirable to ascertain whether the toxicity of Pyridine was due to its possessing basic properties either acting directly or indirectly by its effect upon the *p*H value of the medium. Two sets of flasks were used. One set contained gradually increasing doses of pure Pyridine as in the previous experiment, but in this case starting with ·318 per cent. of this compound (=·004 gm.-mol. per 100 c.c.) and working up by smaller increases to ·636 per cent. The quantities of Pyridine in the second set exactly tallied with those in the first except that before addition the Pyridine solution was brought to a *p*H value of about 4·7 (the same as the medium) by the addition of appropriate amounts of standard sulphuric acid. The culture of *Asp. niger* used was one kindly given to us by the Pure Culture Laboratory at the Lister Institute, No. 594, grown on Czapek's medium and about 7 days old. It proved, unfortunately, rather more susceptible to poison than the one used in the previous test. After inoculation the flasks were set aside in a dark cellar, the temperature of which remained somewhere between 18·5° C. and 19·5° C. Recourse was had to a rather lower temperature as there

appeared to be some escape of Pyridine when the cultures were incubated at 25° C. The yields were weighed after a period of three weeks. An inspection of Table VIII brings out with startling clearness the large differences in yield that ensue through the minimising of the toxic effect of Pyridine by the addition of amounts of acid in quantities sufficient partially to neutralise the base.

Table VIII.

Toxicity of Pyridine to Aspergillus niger.

Culture solution Coons' double strength.
Pure Pyridine S.G. ·983 added.
Culture and organism. *Aspergillus niger* from Lister Inst. No. 594.
Period of incubation 21 days.
Temperature of incubation 18°–19° C.

No.	Description of test	Gm. Pyridine added per 100 c.c. medium	Gm.-mol. Pyridine added per 100 c.c.	Yield of fungus gm.
C 1.	Control (no Pyridine)	—	—	·2881
C 2.	Control „	—	—	·2655
C 3.	Control „	—	—	·2389
P.a. 1.	Pyridine added and acid to adjust *p*H to 4·7	·31115	·00394	·2102
2.	„ „ „ „ „	·3351	·00424	·2164
3.	„ „ „ „ „	·3590	·00454	·2051
4.	„ „ „ „ „	·3829	·00484	·1885
5.	„ „ „ „ „	·4069	·00515	·2293
6.	„ „ „ „ „	·4308	·00545	·2242
7.	„ „ „ „ „	·4547	·00575	·1922
8.	„ „ „ „ „	·4787	·00606	·2119
9.	„ „ „ „ „	·5027	·00636	·2205
10.	„ „ „ „ „	·5265	·00666	·1218
11.	„ „ „ „ „	·5505	·00696	·1666
12.	„ „ „ „ „	·5744	·00727	·1792
13.	„ „ „ „ „	·5983	·00757	·1320
P. 1.	Pyridine added. No adjustment of *p*H	·31115	·00394	·0361
2.	„ „ „ „ „	·3351	·00424	·0212
3.	„ „ „ „ „	·3590	·00454	·0070
4.	„ „ „ „ „	·3829	·00484	·0004
5.	„ „ „ „ „	·4069	·00515	·0002

As we wished to trace the toxic action of Pyridine completely, and as these results indicated that our initial additions of Pyridine were too large, a fresh series of experiments was set up commencing with an extremely small dose (about ·005 per cent.) and ranging up by small additions to concentrations that experience showed were sufficient to inhibit growth completely. The greatest care was taken to ensure that

Table IX.

Toxicity of Pyridine to Aspergillus niger.

Series III. Culture medium Coons' double strength 200 c.c.
Pure Pyridine (S.G. = ·982) added from capillary pipette.
Culture and organism *Aspergillus niger* from Lister Inst. No. 594. Subculture on Coons' agar.
Age of culture 20 days. Inoculated in 5 c.c. sterile water.
Time of incubation 21 days.
Temperature of incubation 18°–19° C

No.	c.c. of Pyridine per 205 c.c.	Gm. Pyridine per 100 c.c. (originally added)	Gm. Pyridine per 100 c.c. found at end of expt.	Mean gm. Pyridine per 100 c.c.	Gm. yield of fungus	Yield after the addition of standard acid to neutralise Pyridine not re-inoculated
1.	·01	·0048	—	(·0048)	·5074	—
2.	·05	·024	·024	·024	·5251	—
3.	·1	·048	·047	·048	·4817	—
4.	·15	·073	—	(·073)	·4887	—
5.	·2	·095	·096	·095	·4717	—
6.	·25	·119	—	(·119)	·4674	—
7.	·3	·143	·136	·139	·4885	—
8.	·35	·167	—	(·167)	·4644	—
9.	·4	·191	·20	·195	·4676	—
10.	·46	·219	·208	·213	·3899	—
11.	·492	·235	·209	·222	·4540	—
12.	·540	·257	·248	·252	·2699	—
13.	·590	·282	·270	·276	·2103	—
14.	·640	·305	·283	·294	·0862	—
15.	·688	·329	·285	·307	·0218	—
16.	·738	·351	·329	·340	·0152	—
17.	·781	·373	·326	·349	·0091	—
18.	·836	·398	·367	·382	·0034	—
19.	·784	·421	(·3845)[1]	—	Traces	·4857
20.	·934	·445	—	—	„	·5185
21.	·984	·468	—	—	No growth	·5424
22.	1·034	·491	—	—	„	·5042
23.	1·082	·515	—	—	„	·5569
24.	1·132	·539	—	—	„	·5250
25.	1·18	·561	—	—	„	·4957
26.	1·23	·585	—	—	„	·5346
27.	1·28	·608	(·558)[1]	—	„	·4811
28.	Control 1[2]	—	—	—	·5604	—
29.	„ 2	—	—	—	·5229	Mean
30.	„ 3	—	—	—	·5390	·5404
31.	„ 4	—	—	—	·5231	—
32.	„ 5	—	—	—	·5568	—

[1] Determined after neutralisation of Pyridine by acid and allowing fungus to grow a further three weeks.

[2] Controls gave a precipitate with Iodine in Potass. Iodide equal to ·0025 per cent. Pyridine. This was allowed for.

each lot of medium should be treated in the same way during sterilisation so that no variations in dilution should take place. The flasks were as far as could be judged of the same size and the cotton-wool plugs rolled in the same way and fitting as nearly as possible the necks of the flasks with an equal tightness. Pyridine was added from a capillary pipette, and the flasks inoculated and incubated as in previous series at 18·5°–19·5° C. for a period of 21 days.

At the end of this period they were filtered and after careful washing and drying the yields obtained were weighed. A portion of the filtrate was set aside and the Pyridine estimated by the method of Harvey and Sparks(7) which we had found to give results of fair accuracy. As the medium itself gave a precipitate with Iodine solution in Potassium

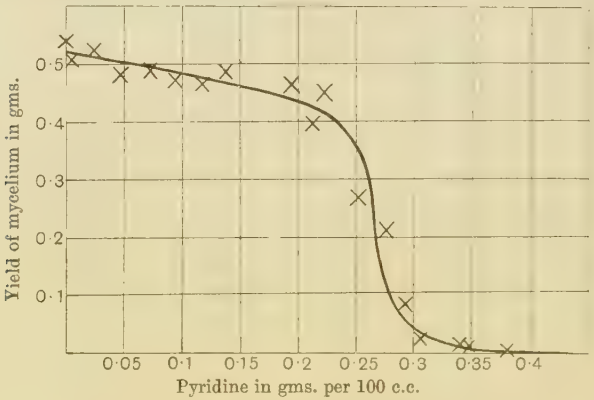

Fig. 3. Toxicity of Pyridine to *Aspergillus niger*.

Iodide, a blank estimation was done on the controls and the amount deducted from that found in the tests. We were thus able to ascertain whether the concentration of Pyridine remained the same throughout the experiment. The means of the amounts of Pyridine added initially and found at the end were taken and plotted against the yields, the curve being drawn freehand through the points.

Table IX and Fig. 3 show that the toxic effect of Pyridine is at first very gradual, the growth yields diminishing very slowly up to a concentration of ·225 per cent. The inhibitive effect of the base from that point onward is, however, increasingly marked and the yields diminish rapidly with small increases in the concentration of Pyridine until at a strength of ·325 per cent. they are almost negligible after which the curve of growth yields tails off very gradually, thus taking a sigmoid shape.

This result corresponds with that obtained by Henderson Smith (4) in his work on the toxic action of phenol on Botrytis spores and it seems probable that a similar explanation should be given in this case, *i.e.* one based upon the variation in resistance of the fungus spores. If it is assumed that this variation is normal and that the spores could be graded according to resistance, then successive grades would contain numbers of spores rising to a maximum in the middle grades and falling again in the last grades. Thus with each successive dose of Pyridine successive grades of spores would fail to germinate. It follows that the middle of the curve is steepest, since with these doses the largest numbers of spores are either killed or their growth inhibited. The toxic effect of the addition of small doses of Pyridine is therefore at first slight then rises to a maximum and falls again as higher concentrations are reached[1].

Table X.

Comparison of Effect of Pyridine and Caustic Soda on growth of Aspergillus *at the same* p*H values.*

No.	Base added	Wt. of base contained in 100 c.c. of medium	*p*H value	Gm. yield of fungus
19.	Pyridine	·429 gm.	6·45	No growth
21.	„	·476 „	6·5	„
23.	„	·524 „	6·52	„
25.	„	·571 „	6·55	„
5.	Caustic soda	·016 „	6·45	·4808
6.	„ „	·018 „	6·5	·4644
7.	„ „	·019 „	6·52	·4792
8.	„ „	·020 „	6·55	·4823
C 1.	Control	—	4·75	·4553
C 2.	„	—	4·75	·4345

[1] As the medium used was slightly acid (*p*H 4·45–4·75) it might be considered that at the lower concentrations of Pyridine its toxicity might be seriously lessened by partial neutralisation. Mr E. M. Crowther kindly determined for us the effect upon the *p*H value of our medium of progressively increasing additions of Pyridine and so the amounts of undissociated base present. With an addition of ·01 gm. of Pyridine to 100 c.c. of medium 53 per cent. was present as free base, while additions from ·1 to ·9 gm. to 100 c.c. of medium gave amounts of free undissociated Pyridine ranging from 82–92 per cent. of the amounts added. The effect upon the curve is to displace it slightly to the left, but not fundamentally to alter its character. We are aware of the fact that *Aspergillus niger* may during growth give rise to notable amounts of acid and that in tracing out an accurate graph of the toxicity of Pyridine to this fungus the amounts of free base before and after the experiment should be determined. This, however, was outside the scope of these preliminary experiments, which were intended to ascertain to what extent a common fungus could tolerate this base when added to a synthetic medium.

As previously stated it was considered that these results might be due to the alteration in *p*H values of the medium on the addition of Pyridine. The medium used has a *p*H value of about 4·75, which the addition of ·429 per cent. of Pyridine brought up to 6·45. To test this point a series of experiments was set up in which the *p*H's of the medium were adjusted by means of *N*/10 Sodium Hydrate to those obtained in the flasks in which the higher concentrations of Pyridine inhibited growth. It will be seen from Table X that the effect of increasing the *p*H from 4·75 to 6·55 is very small. The alteration of *p*H plays, therefore, an insignificant part. One other interesting point arising from these experiments is that the effect of Pyridine is to inhibit the germination of the spores rather than to kill, at any rate all of them, outright. After the yields in Series III, Table IX, had been weighed, additions of standard sulphuric acid were made to the flasks 21–27 where no growth was visible: within two days the spores in these flasks had begun to germinate and growth took place at a rapid rate. After standing for three weeks the yields were weighed, the results being set forth in the last column of Table IX. They are of the same order as those given by the controls during the previous three weeks. At the end of this period the Pyridine remaining in two of the experiments (19 and 27) was determined. The amounts found are expressed in brackets in column 4.

Lutz (6) has stated that in the presence of some other form of assimilable nitrogen, Pyridine may act as a food to fungi. Although our experiments were not set up to investigate this point and must not be regarded as final, for this particular fungus (*Aspergillus niger*) we have not obtained any evidence of Pyridine acting as a stimulant to fungal growth. However small an addition of this base might be made there has never been shown an increase in the yield which could be considered outside the margin of error of the experiment. There is undoubtedly towards the end of the series in Table IX a loss of Pyridine, which however cannot be accounted for by assimilation, being probably due to volatilisation as it is greater as the amount of growth diminishes. Moreover, the deficiency in the amount of Pyridine found after its neutralisation and allowing the *Aspergillus* to grow for a further three weeks is of the same order as that found in the flasks where at the end of three weeks and before neutralisation the growth had been small.

The amount of Pyridine absorbed by culture media from an atmosphere saturated with its vapour is about 4 per cent. in sixteen hours. This is much more than a toxic dose. Subculturing after treatment, especially of fungi growing in liquid media, is therefore essential.

9. DISCUSSION OF RESULTS.

Toxicity of Pyridine and Ammonia to Mites.

Pyridine is shown to have a considerable toxicity to mites and while its effect upon fungi (in the small doses necessary to kill mites) is practically nil, in continually increasing doses it becomes more marked until a concentration is reached at which germination and growth are completely inhibited. The toxicity to mites is surprising as it has generally been assumed that the toxicological action of Pyridine to all living organisms is not marked.

Pyridine and the various monacid ammonium bases have been the subject of considerable toxicological research, either because of their occurrence as groups in the molecular structure of many well-known and widely used alkaloids (*e.g.* Nicotine) or because of their close similarity to them in physiological action.

Brunton and Tunnicliffe(8) have shown that on frogs, Pyridine has, in relatively small doses, a general narcotic action, that its paralysing action on motor nerve endings is of the slightest and that its action is almost wholly confined to the sensory part of the nervous system. They came to the conclusion that Pyridine, compared with its derivatives, is not an active poison, a conclusion that would hardly be expected when the very marked stability of Pyridine is borne in mind. From its close relationship chemically to Nicotine, one would expect a fairly high insecticidal value, yet Pyridine has proved itself of little use in this respect.

Fryer(9) states that after a large number of tests the results have proved in all cases disappointing. The Entomologist to the United States Dept. of Agriculture(10) confirms this and reports that while the compounds most highly poisonous to insects are to be found among the organic nitrogen derivatives the toxic value of Pyridine is small. Tattersfield and Roberts(3) found that to wireworms, Pyridine was less potent as an insecticide than any other of the organic bases tested.

Although the present results do not definitely prove that Pyridine is a compound of *high* specific toxicity to mites, they do indicate that it possesses a toxic action which is much greater than experience would lead us to expect. We were only able to compare it critically with Aniline, a comparison which led to the conclusion that the low vapour pressure of the latter compound tended to put a limit on its toxicity, but that molecule for molecule in the same time it was more poisonous from a quantitative point of view than Pyridine. On the other hand in very minute doses Pyridine had a most profound narcotic effect, inhibiting all the larger movements and leading to almost complete paralysis.

The great toxic action of Ammonia is not surprising, for it is natural to expect such a strongly irritant substance to be highly poisonous to lower forms of animal life.

Toxicity of Ammonia and Pyridine to Fungi.

The toxicity of Pyridine to lower forms of plant life has been the subject of some investigation. The views expressed although somewhat discrepant generally lean towards the opinion of its comparatively low toxic properties. Morgan and Cooper (11) state that of many monacid organic bases they tested the bactericidal properties of Pyridine were the least. Lutz (6) has stated that it may act under certain conditions as a food, a conclusion not borne out by the experiments described above, but it must be recognised that very special conditions as to media and organism may be required for the feeding effect of Pyridine to manifest itself.

Our experiments do not definitely indicate the position of Pyridine in the toxic scale as far as fungi are concerned, but we lean to the view that it is not high. This is not easy to understand, for the compound is inert and its basic properties weak. The latter fact, if the views of Newton Harvey (12) are correct, should indicate a rather high toxicity. This investigator points out that weak bases penetrate cell walls with greater rapidity than strong bases such as Caustic Soda, and that penetration is of the first importance in determining toxicity. On the other hand, another important and countervailing factor is dissociation, the least dissociated bases being least toxic.

As Pyridine is a weak base and very slightly dissociated its toxic properties might be low despite its penetrating power. It is outside the purview of the present investigation to explore this problem, but the rate of penetration of cell walls by chemical compounds is one of fundamental importance in the consideration of fungicidal and insecticidal problems and further investigation along these lines is contemplated. Our results show that fairly high doses such as ·5–·6 per cent. of Pyridine may inhibit germination and growth, and it is probable, although no proof is here advanced, that this is due to the Pyridine readily permeating the cell. These spores, however, will grow if the base is neutralised by acid, the Pyridine in all probability diffusing out of the cell with readiness as soon as the diffusion gradient is modified in a reverse way by the addition of the acid. Our experiments show that what little toxic properties Pyridine may have, it possesses chiefly in virtue of its basic nature. Its salts are hardly poisonous at all either because the acid ion prevents the migration and penetration of the cell wall by the pyridineum ion, or if the salt of Pyridine does penetrate its toxic properties within

the cell are very slight. The toxicity of Pyridine does not arise out of its modification of the *p*H value of the medium but would seem in some way to depend upon a special relationship of the cell to the Pyridine molecule as a base.

10. PRACTICAL APPLICATION OF METHOD.

The following method has been used in the treatment of mite infested fungus cultures with Pyridine. A large bell-jar of about 20 litres capacity is inverted and in the bottom is placed a flat dish containing about 20 c.c. of commercial Pyridine and covered by a wire gauze. The infested cultures, without removing the cotton-wool plugs, are placed in the bell-jar for 16 hours (overnight) and the jar is closed with a glass plate which should be luted down with clay or plasticine. Subcultures taken from the tubes after the above treatment have proved free from the infesting mites, except in one example described above, where some eggs appear to have survived the above treatment, so that in the case of very bad infestations or in very cold weather it may be advisable either to expose the tubes for 48 hours or to give two exposures of 16 hours duration separated by a period of fourteen to sixteen days. The latter method allows any unkilled eggs to hatch, the very susceptible larvae being rapidly poisoned by the second exposure to the vapour of Pyridine. Owing to the rather disagreeable odour of Pyridine it is advisable to carry out the treatment either in a good fume cupboard or outside the laboratory.

Strong Ammonia can be used for cleaning out laboratory apparatus. Its toxic properties to mites are exceedingly great, but as it has a slight but definitely deleterious effect upon some fungi, it is advisable to limit its use to apparatus when its vapour will not play for any prolonged period upon mycological cultures.

Our best thanks are due to Mr H. M. Morris, M.Sc., for much valuable advice and for identifying the species of mites, and to Mr E. M. Crowther, M.Sc., for the determination of the *p*H values of our media.

11. SUMMARY AND CONCLUSIONS.

1. Mites are a serious pest of fungus cultures. The species that most frequently occur are *Aleurobius farinae* and *Tyroglyphus longior* with an occasional infestation with *Glyciphagus cadaverum*.

2. They can be controlled by exposing the cultures to the vapour of Pyridine, after which treatment the fungi can be subcultured safely. An exact description of the application of the method is given on p. 239. (Commercial Pyridine is as effective as the pure material.)

3. If these pests occur in laboratory apparatus they can be eliminated by the application of strong Ammonia. Ammonia and its vapour are very rapidly effective against mites, but they should not be allowed to come into contact with cultures of fungi for too long a period of time in too high a concentration.

4. Pyridine is shown to have a slight toxic action to fungi, and to inhibit growth completely in certain concentrations which, however, are not at all likely to be objectionable in practice, especially if the treated cultures are subcultured.

5. A brief analysis of the toxic action of Pyridine on both Mites and Fungi is given.

(*a*) In the case of Mites minute doses have so powerful a paralysing action as to render it probable that Pyridine is specific in its toxic effect to these pests.

(*b*) In the case of Fungi, the action of Pyridine upon the germination and growth of *Aspergillus niger* was closely studied. It is shown that up to about ·25 per cent., Pyridine has apparently very little toxic action and no feeding effect, but that above this concentration the toxicity increases with great rapidity. It is shown, however, that the toxic action is one of inhibition of germination and that the neutralisation of the base up to 0·6 per cent., the highest concentration tested by us (even though spores have been exposed to its action for three weeks), permits growth to take place rapidly. Pyridine acts chiefly as a poison through its basic properties but not by the change in the *p*H of the medium which ensues on its addition.

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ON THE DEVELOPMENT OF A STANDARDISED AGAR MEDIUM FOR COUNTING SOIL BACTERIA, WITH ESPECIAL REGARD TO THE REPRESSION OF SPREADING COLONIES[1]

By H. G. THORNTON.

(*From the Bacteriological Department, Rothamsted Experimental Station.*)

(With 13 Text-figures.)

1. Introduction.

The recent developments in our knowledge of soil organisms emphasise the necessity for quantitative methods of research on this subject.

An accurate method of estimating the rise and fall of bacterial numbers in the soil, must underlie the study of soil micro-organisms, in their relations both to each other and to the fertility of the soil. The present work was undertaken with the object of increasing the accuracy of the plate method for counting soil bacteria.

When considering this method, it is necessary to bear in mind what kind of information should be obtainable by its use. The method has strict limitations. Thus, not all the physiological groups of soil bacteria are able to develop and produce colonies on any single medium. Yet the medium used should enable the great majority of bacteria in the soil to form colonies upon it, and in speaking of bacterial numbers we usually recognise the exclusion of those few groups which need special media. There are, however, other sources of error in the method, which still further reduce the number of colonies which appear on the platings. Chief amongst these would seem to be the adherence of bacteria to soil particles, the death of some of the organisms during the diluting and plating processes, and the interference between colonies on the plate. The under-estimation of bacterial numbers obtained by the plate method is well shown by work, such as that of Breed and Stocking(1) on the

[1] A grant in aid of publication has been received for this communication.

bacterial numbers in milk, in which the numbers obtained by the plate method were compared with those derived from direct counts made under the microscope. The plate method, therefore, cannot tell us the total numbers of bacteria in a soil sample, but it affords a means of comparing the numbers in two or more samples, by enabling us to count a percentage of the total numbers. To make comparison possible, this percentage must not appreciably vary.

The sources of error in the method, which are not preventable, must therefore be standardised, so that they will affect the calculated numbers to a constant degree. Thus, when comparing the bacterial numbers in different soil types by this method, errors may be introduced by the adherence of groups of bacteria to soil particles. The extent to which this occurs is not at present known, but it is likely to vary in different types of soil. In working with a single soil type, however, this source of error is likely to remain constant and therefore loses its primary importance. The close agreement that we have found(2) between bacterial numbers calculated by this method from parallel soil samples taken at Rothamsted seems to indicate that this is the case. The percentages of organisms which are lost in the diluting and plating process, can be rendered sufficiently constant by careful standardisation of the technique. A variation in numbers obtained, resulting from random sampling, is necessarily involved in making the dilutions. This variation can, however, be calculated and due allowance made for it.

The remaining sources of error are connected with the medium used in the plating and with the development of the colonies therein. The medium appeared to be so great a cause of variation as to render its investigation a matter of first importance, and a necessary prelude to the study of the other factors before mentioned. Its investigation has therefore formed the subject of the present work.

The qualities to be looked for in an ideal count medium have been described by Conn(3). For the purpose of the general bacterial count, constancy in the results obtained with a medium is by far its most important property. The medium should be exactly reproducible by different workers or by the same worker at different times. Also, a suspension of soil, if plated on different samples of the medium, should give rise to the numbers of colonies differing only within the limits of random sampling variance.

Constancy in the results obtained with a medium depends mainly on the following features.

A. The composition of the medium must be constant.

B. There must be as little interference between the developing colonies as possible. For example, the rapid growth of spreading colonies must be checked.

C. The medium must not encourage rapid growth of fungi.

D. Its reaction must vary but slightly.

If the composition of a medium is to be sufficiently constant, it must not contain food constituents whose composition varies. It is in this respect that most of the older media failed, for the earlier work was carried out upon media containing peptone, meat extract, "Nahrstoff Heyden" or some such food supply of uncertain composition.

The first important development from this stage consisted in simplifying the medium and greatly reducing the content of organic matter. Thus Fischer(4) tried a medium containing only soil extract and phosphate as food substances, and Temple(5) used 0·1 per cent. peptone as the sole source of organic matter. It was found that this reduction in organic matter lessened the growth of spreading colonies to some extent and allowed higher counts to be obtained.

At about this time there arose the idea of the "synthetic medium" in which only pure chemical compounds were used as food constituents. Fischer(4) describes such a medium, and Lipman and Brown(6) tried agar media containing dextrose as the source of energy material and KNO_3 of $(NH_4)_3SO_4$ as the nitrogen supply. The medium which they finally developed, however, contained peptone and thus was not truly a "synthetic medium." Brown also tried media with casein, urea, albumen, and asparagine as sources of nitrogen. With the same idea, Conn(3), in 1914, developed an agar medium to which nitrogen was added as ammonium phosphate and sodium asparaginate.

Although past work has thus shown that food substances can be provided in the form of definite chemical compounds, there is great difficulty in obtaining a gel-producing constituent of constant composition. Silicic acid is unsuitable for general use for this purpose. The present author carried out some experiments with cerium hydrate gel, at the suggestion of Dr Emil Hatschek, but was not successful in using it for plating. It would, therefore, appear that an organic colloid such as agar or gelatine is alone suitable for this purpose. In the constancy of the results obtained with it, agar is far superior to gelatine, both on account of its less variable composition and because of its comparatively low feeding value to bacteria. This is illustrated in the following experiment (see Table I). Media were made up in which the food constituents of Conn's sodium asparaginate medium(3), were added to four different

brands of agar and three different brands of gelatine. The agar media were sterilised in the autoclave at 15 lbs. for 15 minutes and the gelatine for 20 minutes at 100° C. on three consecutive days. The media A and B were made up with the same agar, all the constituents being separately weighed out in each case, in order to test the error involved in preparing the media. This error appears to be negligible. The media were tested with regard to their acidity and their capacity for colony development.

The H-ion concentration was measured by the indicator method of Clark and Lubs, both before and after sterilisation.

The greater constancy of agar is noticeable both in the original reaction and in the smaller change which occurs on sterilisation. To test the colony development, six parallel platings were poured with each medium, from a single diluted suspension of Rothamsted soil. The results again show the advantage of agar in that it is less variable in its effects, a feature evidently connected with the greater constancy in reaction between samples.

Table I.

Variability between Samples of Agar and Gelatine.

Medium	Sample of agar or gelatine	*p*H value before sterilising	*p*H value after sterilising	Bacterial colonies on each plate	Mean no. of colonies
A.	Sample 1. Shred agar	6·7	6·6	33, 32, 31, 30, 30, 28	30·7
B.	Same. Salts weighed separately	6·7	6·6	34, 33, 32, 30, 30, 29	31·3
C.	Sample 2. Shred agar	6·8	6·65	47, 35, 35, 34, 33, 27	35·2
D.	„ 3. Powdered agar	6·8	6·6	35, 34, 34, 33, 28, 27	31·8
E	„ 4. Powdered agar	6·8	6·5	50, 46, 45, 42, 40, 38	43·5
F.	„ 5. Gelatine	6·0	5·7	16, 14, 14, 12, 10, 6	12·0
G.	„ 6. Gelatine	6·4	5·9	15, 13, 13, 12, 11, 8	12·0
H.	„ 7. Gelatine	5·4	5·2	8, 7, 6, 5, 5, 4,	5·8

Sample E in the above experiment was an obviously impure agar powder such as would not have been used in routine work. The somewhat abnormal results obtained with it, however, show the advisability of employing some method of washing the agar before use. Fellers(7) found that agar contained compounds of Ca, Mg, S and N which were soluble in 0·5 per cent. HCl. He also observed that agar could support a slight growth of bacteria which produced ammonia therefrom. Several methods of washing and purifying agar have been tried by various authors. Thus Fellers(7) made a sol of 5 per cent. agar in distilled water and precipitated this in alcohol. Cunningham(8) washed agar in dilute acid, filtered it through cotton-wool in an autoclave, and dried the filtered product in an oven. It is claimed that this product is purified and that

filtration of the media prepared from it is greatly facilitated. Most workers, however, have washed the agar either with water or dilute acid and have then dried it, while in the shred condition. To test the advantages of washed agar in counting technique, some agar was washed and filtered by Cunningham's method, and a second quantity was washed while in the shred condition in 0·1 per cent. H_2SO_4 for 10 minutes at 15° C., rinsed free from acid by continued changes of water, and dried. A medium, having the following composition, was made up with the two samples of agar and with unwashed agar as a control.

Distilled water	1000 c.c.	CaCl ...	0·1 gm.
Agar ...	15 gm.	KCl ...	0·1 ,,
K_2HPO_4 ...	1 ,,	Dextrose ...	0·5 ,,
$MgSO_4.7H_2O$	0·2 ,,	Asparagine ...	0·5 ,

Each of the three media was tested (*A*) without filtering, (*B*) after filtering at 100° C., (*C*) with the salts filtered before adding the agar, which was not filtered. The acidity was adjusted before autoclaving. In each medium the change of reaction during sterilisation in the autoclave was tested, and platings of a single diluted suspension of Rothamsted soil were made to test its capacity for allowing colony development. The data obtained are shown in Table II.

Table II.

Effect of Acid washed Agar.

Treatment	*p*H value before adjustment	*p*H value adjusted to	*p*H value after autoclaving	Bacterial colonies on each plate	Mean no. of colonies
Agar unwashed, medium not filtered	6·7	7·3	7·0	90, 87, 85, 82, 78, 73	82·5
Agar unwashed, medium filtered	6·7	7·3	7·0	74, 74, 72, 69, 65, 63	69·5
Agar unwashed, salts filtered	6·7	7·3	7·0	95, 80, 72, 70, 68, 68	75·5
Cunningham treatment, medium not filtered ...	6·45	7·2	6·7	53, 52, 52, 48, 47, Sp.*	50·4
Cunningham treatment, medium filtered ...	6·45	7·2	6·7	69, 63, 54, Sp. Sp. Sp.	62·0
Cunningham treatment, salts filtered	6·4	7·2	6·7	62, 61, 55, 53, Sp. Sp.	57·8
Acid washed and dried, medium not filtered ...	6·6	7·2	6·8	82, 75, 70, 68, 65, 58	69·6
Acid washed and dried, medium filtered ...	7·2	7·2	6·9	83, 77, 73, 71, 69, 58	71·8
Acid washed and dried, salts filtered	6·5	7·2	6·8	80, 76, 75, 64, 60, 59	69·0

* Sp.: platings lost through spreading organisms.

It will be seen that Cunningham's method of washing and filtering has produced undesirable changes in the agar. The alteration in reaction during autoclaving has been increased, while the number of colonies which develop is distinctly lowered and spreading colonies are encouraged.

Taking the media made up with agar washed in the shred condition it will be seen that in the filtered medium, the colony development is unaffected by the washing in acid but that when the medium is not filtered the unwashed agar permits a rather higher number of colonies to grow. In making up the medium for routine work, however, filtering is necessary, so that the washing will not produce a harmful effect on the development of colonies. As a result of many trials it has been found that agar washed in acid, while in the shred condition, gives more regular results than unwashed agar.

The process of washing in acid has the further advantage, also observed by Cunningham (8), when using his method, that it renders filtration of the medium easier and more rapid. For example, the medium used in the last experiment was made up with unwashed agar and with shred agar washed in 0·1 per cent. acid. The time taken to filter 200 c.c. of medium through filter paper in a warm filter funnel was recorded. It was found that washed agar medium passed through the filter paper in 55 minutes while the unwashed agar medium took 2 hours to pass through.

Experiments with various strengths of acid for use in the washing have resulted in the adoption of a routine technique in which the agar shred is soaked in 0·05 per cent. H_2SO_4[1] for 15 minutes at room temperature, washed in water till acid free, and then dried.

Although the difference in composition between samples of agar may be lessened by washing in acid, yet the removal of impurities is not complete. The effect of these varying impurities must therefore be neutralised. Small quantities of organic impurities, such as occur in the washed agar, are unlikely to influence bacterial growth in a medium already richly supplied with organic and nitrogenous food substances. In a similar manner, the influence of traces of Ca, Mg, S, etc., may be masked by the addition to the medium of quantities of these substances in excess of the bacterial requirement. The necessity of this addition in a medium from which standard results are expected, is sometimes overlooked. This is the case, for example, in some of the "simple" media that have been

[1] For some time 0·5 per cent. acid was used, but it was sometimes found that this affected the gel formation of the agar.

used both for bacteria and fungi, where reliance is placed on such variable impurities as may be present in the agar, to supply the electrolytes needed for growth. In the present work the Ca, Mg and S salts used in Conn's sodium asparaginate agar(3) have been employed with the addition of NaCl as a source of sodium.

2. The Spreading Growth of Organisms on Agar Plates.

Unfortunately agar, when used in a count medium, has one defect that is so serious as to have deterred some workers from its use. Certain commonly occurring soil bacteria form rapidly spreading surface colonies on agar, which, in many platings, cover the agar surface and inhibit or interfere with the development of other colonies. These organisms are so abundant in Rothamsted soil that on meat-extract peptone agar a large percentage of platings are spoilt, and accurate bacterial counts are impossible on such a medium. If the amount of organic nitrogen compounds in the medium be reduced, there is less growth of the spreading organisms. The fact has long been realised and led to the development of such media as Lipman and Brown's agar medium(6), containing only 0·05 per cent. peptone. However, considerable "spreading" still takes place on such media as the above. Conn(3) noticed this fact, which I have also observed with this and with other peptone media. Less "spreading" seemed to occur on media containing simpler organic nitrogen compounds, such as Conn's sodium asparaginate agar. This indicates that a mere reduction in the amount of organic nitrogen in the medium is not an efficient means of checking spreading colonies, but that the nature of the compounds used is of importance. A more exact knowledge of the conditions which control the growth of spreading colonies, and especially of the effect on them of the composition of the medium, appeared necessary.

It was therefore decided to study the behaviour in pure culture of an organism which formed spreading colonies, in the hope that the knowledge thus obtained would enable a medium to be developed upon which the formation of spreading colonies would be restricted. By far the most abundant of these spreading organisms in Rothamsted soil is spore-forming bacillus which appears to be similar to *B. dendroides* described by Holzmuller(9) in his paper "Die Gruppe des Bacillus mycoides." The organism, however, would appear rather to belong to the *B. subtilis* group. The strain here used has the following characters[1].

[1] I am indebted to Mr P. H. H. Gray for having worked out the characters of the organism in this laboratory.

Morphology. (A) *Vegetative Cells.* (Nutrient agar, Conn, 2 days incubation at 20° C.) The organism consists of short rods, lying singly or in pairs and short chains. Some long rods, up to 10μ occur. Size of majority 4 × 0·5μ. The rods are actively motile, and bear 6 to 15 long undulating flagella, which are peritrichous. The organism is gram positive, and takes readily all the usual stains.

(B) *Spore formation.* (Nutrient agar, 4 days at 20° C.) Sporangia consist of slightly thickened rods often in chains. Endospores central in position, elliptical, size of majority 1·25 × 0·75μ.

Cultural Characters. (A) *Agar stroke.* (2 days at 30° C.) At first filiform, later (4 days), flat and spreading. Smooth glistening surface. Growth opaque and whitish.

(B) *Gelatine stab.* (2 days at 20° C.) Growth best at surface. Line of puncture filiform. Liquefaction commences in 2 days and becomes napiform. After 30 days, depth of liquefaction is about 25 mm.

(C) *Potato.* (2 days at 30° C.) Abundant, viscid growth of whitish colour. Surface dull and wrinkled. Potato discoloured brown.

(D) *Nutrient Broth.* (2 days at 35° C.) Surface pellicle formed. Liquid slightly clouded. No sediment. No odour.

(E) *Agar Colonies.* (2 days at 30° C.) Very rapid growth. Colony formation described below.

(F) *Gelatine Colonies.* (2 days at 20° C.) Circular with entire edges. Saucer-shaped liquefaction. Rather slow growth.

Physiology. (A) *Fermentation.* (Fermentation tubes containing nutrient broth + 1 per cent. of the compound indicated. Incubation 4 days at 30° C.)

Dextrose. Reaction acid (no gas).
Saccharose. Reaction acid (no gas).
Lactose. Reaction alkaline (no gas).
Glycerine. Reaction alkaline (no gas).

(B) *Diastatic Action.* (Starch agar plates incubated at 30° C.) Action strong. Breadth of clear zone in 5 days, 5 to 10 mm.

(C) *Litmus Milk.* (10 days at 30° C.) No coagulation. No change in reaction or litmus reduction.

(D) *Indol Formation.* Negative.

(E) *Nitrate Reduction.* (Nutrient broth + 0·1 per cent. KNO_3 incubated at 30° C.) Nitrite present in two days—no gas in 10 days.

(F) *Chromogenesis.* Negative.

(G) The organism is aerobic. Its optimum temperature for growth is about 35° C.

Before investigating the action of varied external conditions on the "spreading," the process by which the organism produces the normal spreading colony on the surface of meat-extract peptone agar was carefully studied. The germination of spores and the early stages in the division and grouping of the cells were observed by means of the agar block technique described by Hill (10).

In order to observe the formation and subsequent spreading of a colony, platings of sterile nutrient agar were poured and were inoculated at the surface with spores of *B. dendroides*. The plates were incubated at 37° C., and were examined at short intervals under a $\frac{1}{6}$-inch objective. In this way the development of a surface colony could be observed on the actual plating. Owing to the extreme rapidity with which *B. dendroides* grows, no difficulty was experienced from air contaminations, which had no time to develop during the short period concerned.

When incubated at 37° C. on the surface of nutrient agar, the spores germinate in about 45 minutes, producing rods which are at first non-motile. The rods divide rapidly and the daughter cells do not form chains but come to lie side by side so as to form packets of four to six cells. A young colony at this stage has mosaic-like appearance under a low power, owing to the packets of rods lying at divergent angles. The formation of these packets is not uncommon in other organisms and is described and illustrated by a number of authors. Their production is probably conditioned by surface tension and has an important influence on the development of the colony.

At about this time a water film, covering the colony, becomes noticeable. It seems probable that this is derived from the saturated atmosphere covering the agar film, the young colony acting as a point upon which condensation occurs. The surface growth of the organism possesses the power of retarding the absorption of this water by the underlying agar. The following experiment illustrates this. Droplets of distilled water were placed on a portion of the agar over which a colony was spreading and other drops of similar volume upon a sterile portion of the plating. The former took four to six times as long to disappear as the latter. The time taken for water to be absorbed was found to vary according to the thickness of the agar film and other conditions but was always several times greater within the area of a branching colony than outside it. The absorption of water within the colony area was found to be retarded not only where the surface was entirely covered with bacterial growth but also in places where a large fraction of the surface consisted of apparently uncovered agar lying between branches of the

colony. It seems, therefore, that the retention of the water cannot be due merely to the closely packed bacteria separating it from the underlying agar.

Two explanations of these facts suggest themselves. Either substances that hinder the access of the water to the agar may cover the surface in neighbourhood of the bacterial growth, or else the organisms may produce a local change in the agar gel such that its capacity to absorb water is reduced. The latter hypothesis can be tested by growing the organism on the lower surface of a film of agar and measuring the rate of absorption of water drops by the agar immediately above the growth and elsewhere. Under these conditions, any substances produced by the organism on the surface of growth will no longer lie between the agar and the water drops, whereas a change in the absorption capacity of the agar would reveal itself, and, if found to occur, the depth to which the gel is affected could be observed by varying the thickness of the agar film. This test was applied by the following experiment. A very small droplet of 12 per cent. gelatine was placed in the centre of each of six sterile petri dishes, and each droplet was inoculated with spores of *B. dendroides* and allowed to set. Nutrient agar medium, melted and cooled to 42° C., was then poured into each dish. The gelatine kept the spores adhering to the glass so that all subsequent growth of the organism took place beneath the agar film. By varying the quantity of agar in the dish, the thickness of the film was varied. Quantities of from 5 to 20 c.c. were used, giving films of from about 1 to 3 mm. in thickness. After about 48 hours incubation there was good growth along the bottom of each plating. Drops of distilled water, 0·02 c.c. in volume, were placed on the agar surface in each dish, both above the bacterial growth, and outside the colony area, and their rates of absorption by the agar were measured. On all the platings, the water was absorbed at an equal rate above the bacterial growth and outside this area. It appears, therefore, that there is no alteration in the water absorbing capacity of the agar gel in the neighbourhood of the colony. We must therefore conclude that the retention of moisture on the surface about the bacterial growth is due to some secretion, probably of a mucilaginous nature which hinders access of the water to the underlying agar.

The rods, that are at first produced by germination of the spores, are non-motile, but soon peritrichous flagella are developed. These have mean length of about 10 μ, and are undulating. In the young colony the cells bear 8 to 15 flagella, but this number appears to be reduced to about half in the older growths.

As the moisture appears, a slow motility can be observed in the colony, the rods sliding over one another and slowly pushing outward the edge of the colony. Single rods do not appear capable of overcoming the surface tension at the edge of the water film. They have many times been observed pushing outward but do not force their way out from the colony[1]. Packets of six or more cells, however, are able slowly to press outward the edge of the water film. Consequently, where a packet of cells lies at the edge of the colony, in such a manner that the rods lie radially, or at right angles to the film edge, they may often be observed

Fig. 1. *Bacillus dendroides.* Successive stages in the formation of a process from a 6 hours old surface colony on nutrient agar.

to force their way outward producing a small promontory (Fig. 1). But where the outer packets lie so that the cells are oriented tangentially, they do not press away from the centre. The out-pushing of the colony edge is therefore discontinuous so that the colony becomes irregular or lobate. As the water film becomes thicker, the cells move about more actively, and, in the interior of the colony, they often lose their arrangement in packets, and where there is most moisture a streaming movement of cells may be observed. In each projection of the colony edge, the cells tend to swim outward and to collect at the distal extremity,

[1] An attempt was made to modify this condition by adding 0·05 per cent. saponin to the medium in order to lower the surface tension of the film. The saponin, however, caused abnormal growth of the organism.

where by their multiplication and further outward movement they further extend the projection. In this manner, the colony is produced into radiating branches (Fig. 2). In a branch, cells can be seen moving toward the outward extremity so that the proximal region of the branch

Fig. 2. *B. dendroides.* Young colony, diameter about 1 cm.

Fig. 3. *B. dendroides.* Young process from a surface colony (nutrient agar 12 hrs. 20° C.).

soon becomes partially depleted of cells, in many places only scattered isolated cells remaining. Towards the outer end the cells are packed close together and near the tip of the process they are piled up, two or more layers of cells overlying one another (Fig. 3). This arrangement can be

seen in the living condition and has also been studied in microtome sections of the processes, prepared by the technique developed by Legroux and Magrou (11). It is uncertain whether the outward movement of the cells is due to the repelling influence of substances produced at the centre of the colony or to attraction by some factor in the medium outside.

The branching colony thus produced, spreads over the surface at a surprising rate. Thus, on a nutrient agar plate incubated at 37° C. a colony has been observed to increase in diameter from 0·6 cm. to 1·75 cm. within two hours.

Under conditions favourable to the organism, the spreading growth may continue until the entire surface of the plating is covered with growth. As a rule, however, if the area of a spreading colony be measured at intervals, it is found that a short lag period is followed by rapid spreading expansion which soon becomes increasingly slower, until it finally ceases after a period which varies according to the conditions of growth.

On nutrient agar, the cessation of spreading is accompanied by a change in the morphology of the organisms. Long chains of cells are produced within which endospores soon appear. The formation of these chains of sporangia produces a marked alteration in the appearance of the colony under a low power. At the tips of the branches, the edge is now composed of a mass of convoluted filaments, similar in appearance to those that compose colonies of *Bacillus anthracis*. Growth at the extremity of the branch now takes place laterally, so that the tip expands and becomes club-shaped. In the interior of the colony no further growth occurs, the cells forming endospores.

With the object of discovering a means of checking spreading growth it was important to ascertain the cause which normally brings about the slowing and final cessation of spreading. In the course of many experiments, it has been found that the length of time during which spreading continues is not noticeably affected by the quantity or nature of the food constituents of the medium, although, as will be shown, the amount of spread occurring within that period is greatly influenced by these factors. This suggested that the cause was not nutritional but rather of a physical nature. From our knowledge of the method by which the spreading occurs, through actual motility of the cells in the moisture film, it seemed probable that the cessation of spread was due to the evaporation of this surface moisture. That the drying up of this water film is able to arrest the spreading was shown by the following experiment in which

agar platings were dried for various periods before inoculation with the spreading organism. Platings of sterile nutrient agar were poured and were dried for periods of 14, 11, and 2 days respectively and control platings were dried for two hours. Five parallel platings were prepared for each period of drying. The drying took place in an incubator at 30° C. All the platings were then inoculated at the same time with 0·02 c.c. of a suspension of *B. dendroides* spores, and were incubated at 30° C. The growth after 48 hours is recorded in Table III.

Table III.

Spreading of Bacillus dendroides. *Effect of previous drying of plates.*

Period of drying days	Area of growth in 48 hours sq. cm.
14	0·8
11	1·2
2	2·0
Nil	51·6

It will be seen that the spreading is checked by drying of the water film even when this takes place before inoculation. On the plates that had been dried for two or more days, the period of motile spreading was entirely inhibited, and the organism developed long chains of cells in which spores were produced, the colony assuming the anthrax-like edges characteristic of normal growth after spreading has ceased. On the control plates the spreading was quite normal.

Experiments similar to the above have also been carried out, using a "synthetic" agar medium and in this case also the limitation of spreading was observed on platings dried previous to inoculation.

If the retardation and cessation of spreading on platings be normally due to drying of the surface moisture, we should expect that if this drying were prevented, the colony would continue to spread indefinitely, increasing in area at an even rate, without retardation, until the plate was covered. This point was therefore tested by growing *B. dendroides* on platings kept in an atmosphere saturated with moisture.

Six sterile platings of synthetic agar medium were poured and inoculated at the centres with spores of *B. dendroides*. The plates were kept at room temperature, in an atmosphere saturated with moisture in a Novy jar. The areas of growth of the organisms were measured at intervals. Fig. 4 shows the mean areas of growth on the six parallel plates, plotted

against the time of incubation. It will be seen that the growth increases in area at a perfectly even rate there being no retardation in the rate of spread even after eleven days.

The result of this experiment also disposes of the view that the retardation of spreading is the result of an increasing accumulation of metabolic products of growth.

It therefore seems clear that the progressive retardation of spreading observed on normal platings is the effect of drying. This drying could operate either by reducing the film of surface moisture in which spreading takes place or by producing an unfavourable increase in the concentration of salts in the medium. This question can be examined by measuring the rapidity of spreading on media in which the concentration of agar

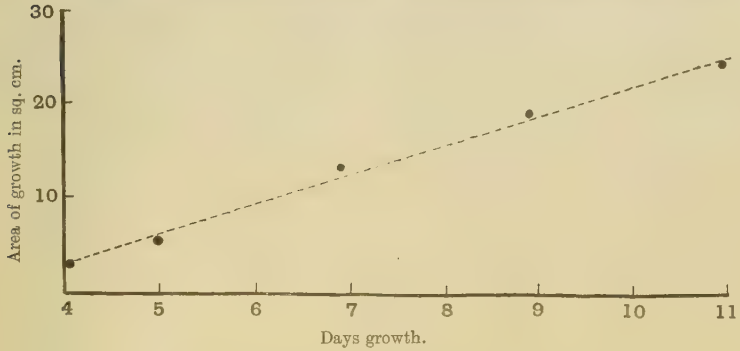

Fig. 4. *B. dendroides.* Spreading over agar plates in a saturated atmosphere.

is varied. An increase in percentage of the agar will not appreciably increase the concentration of salts, but will reduce the relative amount of free water in the medium and in consequence shorten the time taken for the surface moisture film to evaporate. So that if the mere drying of the surface moisture is the cause, the period of spreading should be shortened as the percentage of agar is increased.

A "synthetic" agar medium was therefore made up with three percentages of agar—0·5, 1 and 2. Five platings of each medium were poured and after being kept at 20° C. for 24 hours in order to start the drying, were inoculated with *B. dendroides*. The plates were incubated at 20° C., and the area of growth measured at intervals. The mean areas of growth on the sets of five parallel plates are shown in Fig. 5 in which the area of growth is plotted against the time.

It will be seen that with 2 per cent. agar the spreading ceases within five days, while with 1 per cent. agar it continues for a longer period, although the normal retardation of spreading is well shown.

With 0·5 per cent. agar an interesting result appeared. As will be seen from the curve, the increase in colony area took place more slowly, but there was no falling off of the growth, the increase in area taking place quite steadily till the end of the experiment. This steady increase was accompanied by an entire change in the form of growth. The colony was quite circular, nearly transparent and had an indistinct edge, which

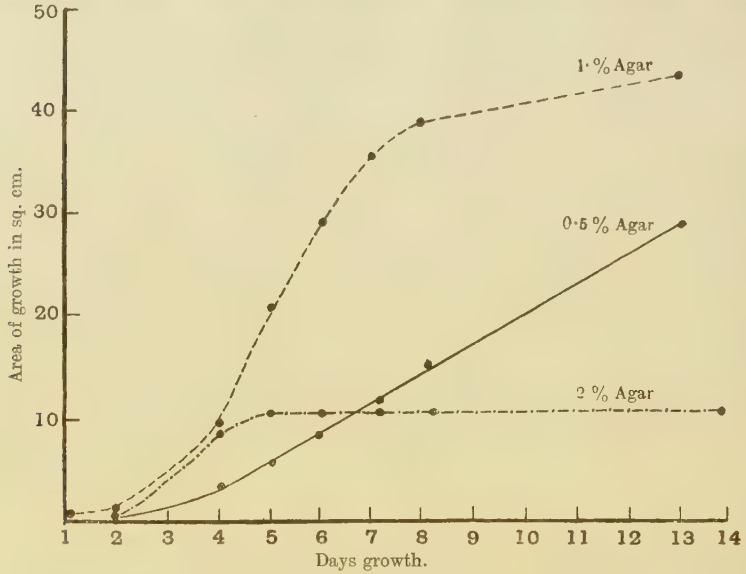

Fig. 5. *B. dendroides*. Spreading growth with varying percentages of agar.

was not produced into processes. The alteration in the mode and form of colony growth was due to the stiffness of the agar having been reduced to a point at which the motile organisms were able to penetrate the substance of the gel and progress slowly through it. When the growth was examined under a $\frac{1}{6}$-inch objective it was found that instead of forming a layer of surface growth, the rods were distributed throughout the agar, each rod lying separately and moving through the gel with a restricted, jerky motion. The colony grows as an ever-widening disc, since the forces which normally lead to the formation of branches do not operate. Also there is no falling off of the rate of growth, since the

limited motility is not dependent on the surface moisture and the evaporation from the gel itself is too slow to produce visible effect.

The experiment thus shows that slowing and cessation of spreading are due normally to the disappearance of the surface moisture film, for they take place after a longer period when the proportion of water to agar is increased, and do not occur if the gel be so thin as to allow the motile organisms to penetrate away from the surface.

As described above, the cessation of motile spreading on nutrient agar is accompanied by the formation of sporangia and endospores. It appeared possible that the composition of the medium might be so altered as to bring about spore formation and consequent loss of motility and spreading, before the water film dried, thus shortening the period of spreading. I therefore investigated the influence of various substances on spore formation in the organisms. Tests were first made with different organic nitrogen compounds. The medium used as a base in these experiments had the following composition:

K_2HPO_4	...	1·0 gm.	KNO_3	...	0·5 gm.
$MgSO_4.7H_2O$	...	0·2 „	Dextrose	...	1·0 „
$CaCl_2$ ...	...	0·1 „	Agar	...	15·0 „
NaCl ...	...	0·1 „	Water	...	1000 c.c.
$FeCl_3$ ...	...	0·002 „			

To this medium, various nitrogen compounds were added in amounts giving nitrogen equivalent to 0·05 gm. of asparagine. In each medium to be tested, duplicate stab cultures of *B. dendroides* were made and incubated for 14 days at 30° C., after which the growth was examined for spores, both alive and by means of Ziehl Neelsen's spore stain. Media containing the following sources of nitrogen were tested.

1. KNO_3 alone.
2. KNO_3 + Alanine.
3. KNO_3 + Asparagine.
4. KNO_3 + Tyrosine.
5. KNO_3 + Peptone.
6. KNO_3 + Lemco.

On the medium without organic matter and on media 2 and 3, to which alanine and asparagine were added, no spores were produced. On platings of such media, surface colonies cease to spread when the surface water dries off, but the rods retain their flagella and active motility is immediately resumed if the surface be wetted. When, however, the stab cultures had been kept for two months at 30° C., it was found that spore formation had taken place on all media. I also found that if platings of *B. dendroides* on the KNO_3-asparagine medium were dried over H_2SO_4, spores were produced after 11 days. Thus spore formation

can take place on these media, but only after much more intense drying than is needed to induce it on nutrient agar. On the media containing peptone, lemco or tyrosine, spores were produced in large numbers within 14 days. It would appear that these substances contain a component which renders spore formation more easily induced. But even on these media, spores were not produced on platings until drying of the surface water had stopped the rapid spread of the organisms. Experiments were also made with various carbohydrates, which, however, were without effect on spore formation. While, therefore, the formation of spores can be retarded by certain conditions of nutrition, it appears on agar platings as a reaction to drying of the surface moisture, and it would seem that it cannot readily be induced until this immediate cause begins to operate[1].

In applying our knowledge of the mode of growth of *B. dendroides*, in an attempt to check its spreading over agar plates, the following facts must be borne in mind.

A. The duration of the period of rapid spreading is limited by the surface moisture of the agar and terminates when this disappears. Methods of drying the agar surface so as to curtail this period do not appear practical in routine work involving a large number of platings. It is not at present possible to shorten the period of spreading by hastening the incidence of spore formation.

B. The rapidity of spreading during the existence of the surface moisture film is influenced by two characters:

(1) The motility of the organism.

(2) Its rate of multiplication.

It has not been found possible to reduce the motility of the organism on platings during this period by any change in the composition of the medium[2]. The rate of multiplication, on the other hand, is greatly influenced by the food supply, and it seemed probable that by checking this during the period of spreading, the area of spread could be much reduced.

It is known that the content of organic matter of the medium influences the formation of spreading colonies on plates (3). I therefore decided to investigate the influence of the organic nitrogen constituent

[1] Cultural conditions liable to inhibit the development of other soil organisms on the plates are not here considered.

[2] H. Braun (16) found that *B. proteus*, if grown on agar media in which the nutrient material and salts were reduced to $\frac{1}{10}$ the normal concentration, lost its flagella and consequently formed non-spreading colonies. Unfavourable food conditions, however, do not appear to influence the motility of *B. dendroides*, though affecting its multiplication rate.

of the medium on the rate of multiplication of *B. dendroides*, comparing its growth on media containing, respectively, peptone, "lemco," and a pure amino-acid.

The medium used as a basis had the following composition:

Medium CV.

K_2HPO_4 ...	1 gm.	$FeCl_3$ ...	0·002 gm.
$MgSO_4.7H_2O$ ...	0·2 ,,	KNO_3 ...	0·5 ,,
$CaCl_2$	0·1 ,,	Mannitol ...	1·0 ,,
NaCl	0·1 ,,	Water ...	1000 c.c.

To the above medium the organic nitrogen supply was added in an amount giving nitrogen approximately equivalent to 0·05 per cent.

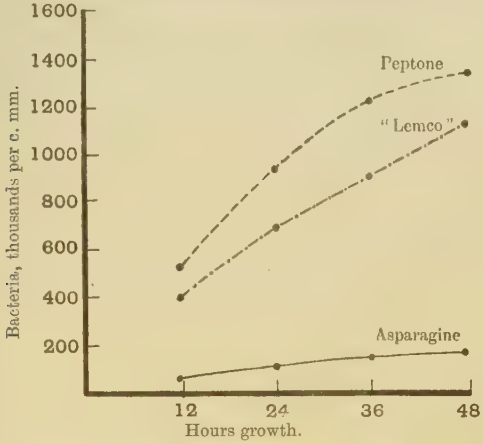

Fig. 6. *B. dendroides*. Effect of the organic nitrogen source on multiplication.

asparagine. The reaction of the media was standardised to *p*H 7·4, immediately prior to autoclaving. Duplicate tubes containing 10 c.c. of medium were inoculated with 0·5 c.c. of a suspension of a young agar culture of *B. dendroides*. The tubes were incubated at 30° C., and, at intervals of 12 hours, the number of organisms per c.c. was estimated from counts made in a Thoma counting chamber, the mean count of the duplicate tubes being taken in each case.

The curves, Fig. 6, show the multiplication of the organisms in media containing "lemco," peptone, and asparagine.

It will be seen that, with asparagine, the multiplication is very markedly less rapid than in the presence of peptone or meat extract.

It therefore appeared probable that the amount of spreading would be correspondingly decreased on a medium containing amino-acid as the source of organic nitrogen. I therefore measured that increase in area of surface growths of *B. dendroides* upon the media employed in the last experiment, made up with 1·5 per cent. agar. A medium was also tested containing equivalent nitrogen in the form of tyrosine. Tubes of each medium were autoclaved and poured into sterile petri dishes. Each

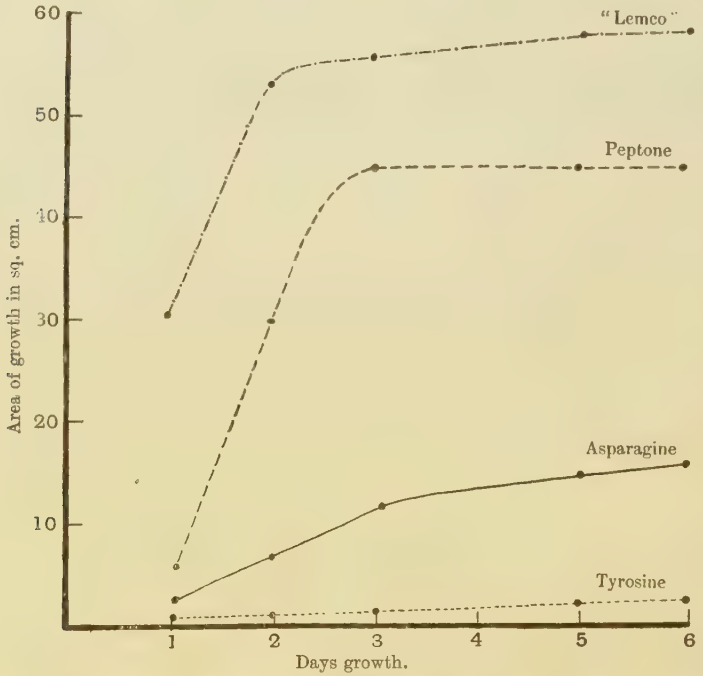

Fig. 7. *B. dendroides.* Effect of the source of organic nitrogen on spreading growth.

plate, when the agar had set, was inoculated at the centre with 0·01 c.c. of a suspension of a 48-hour old culture of *B. dendroides.* The plates were incubated at 20° C., and the area of growth on each plate was measured at intervals, over squared paper. Eight parallel platings of each medium were prepared and the mean areas of growth are shown in Fig. 7, in which the area of growth is plotted against the time.

The curves show that spreading growth is very much reduced when peptone or meat extract is replaced in the medium by a simple amino-

acid. This explains the fact, observed by Conn(3), that on Lipman and Brown's peptone agar, "overgrowths are often so abundant...as to interfere with counting and prevent the isolation of pure cultures from the colonies," and also that these overgrowths are reduced on his medium containing ammonium phosphate and sodium asparaginate.

On the tyrosine medium scarcely any growth took place. It appears that this is due, not to any inhibiting action of the tyrosine, but to the inability of the organism to make full use of the tyrosine molecule in its nutrition. This is shown in the following experiment, in which the

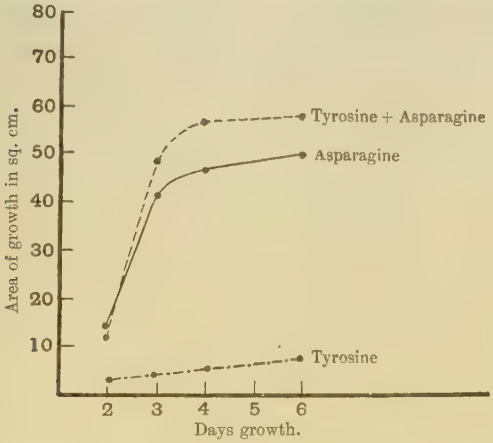

Fig. 8. *B. dendroides.* Effect on tyrosine and asparagine on the spreading growth.

growth was compared on media similar to those tested in the last experiment but having organic nitrogen supplied as follows:

A. 0·12 per cent. tyrosine.

B. 0·12 per cent. tyrosine + 0·05 per cent. asparagine.

C. 0·05 per cent. asparagine.

The test was conducted in a manner similar to the last experiment except that the plates were incubated at 25° C. to accelerate the growth.

The curves (Fig. 8) show the mean areas of growth, measured at intervals, of ten parallel platings in the case of media B and C and of seven parallel platings in the case of medium A. It will be seen that in the presence of asparagine, tyrosine does not check but slightly stimulates the growth, whereas with tyrosine alone the growth is very slight. Tests made with the tyrosine medium, however, showed that it was

unsuitable for use in bacterial count work, owing to the very rapid development of moulds which took place on it.

Tests were also made with media containing glycocoll and alanine. These media were found to give results comparable with but not better than those obtainable with asparagine.

The entire omission of organic nitrogen was found greatly to reduce the spreading, but the number of bacterial colonies which developed from a suspension of Rothamsted soil, when plated on such a medium, was so much reduced by the omission of organic nitrogen, that the medium was unsuitable for counting work.

It was therefore decided to use asparagine as the organic nitrogen supply in the medium, and trials were made to ascertain the concentra-

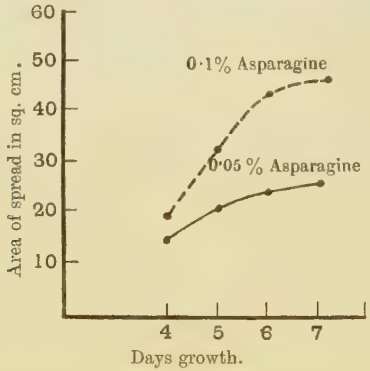

Fig. 9. *B. dendroides*. Effect of asparagine content on spreading.

tion which produced the least spreading of *B. dendroides*, while allowing the best colony development when the medium was used for counting other bacteria. In these trials medium CV (p. 259) made up with 1·5 per cent. agar was used. The technique used to estimate the spreading was similar to that described above. Fig. 9 shows the growth area of *B. dendroides* with 0·1 per cent. and 0·05 per cent. asparagine. The colony development on these two media was also tested, eight parallel platings of a suspension of Broadbalk soil being made on each medium. The colony counts obtained were significantly higher on the medium containing 0·05 per cent. asparagine.

The increase in the asparagine content is thus detrimental as it both favours the spreading of *B. dendroides* and is harmful to the development of other organisms.

The effect of reducing the asparagine content below 0·05 per cent. is shown in Fig. 10, where the increases in area of surface growths of *B. dendroides* on media containing 0·05 per cent., 0·005 per cent. asparagine and no asparagine, are plotted. It will be seen that while the growth is reduced in the total absence of organic nitrogen, the reduction of asparagine content to 0·005 per cent. was without appreciable influence on the spreading.

This reduction, however, was found to produce a significant falling off in the number of colonies developing when a suspension of Rothamsted soil was plated on the two media.

The optimum concentration of asparagine in the count medium is therefore in the region of 0·05 per cent. This concentration has been adopted in the medium.

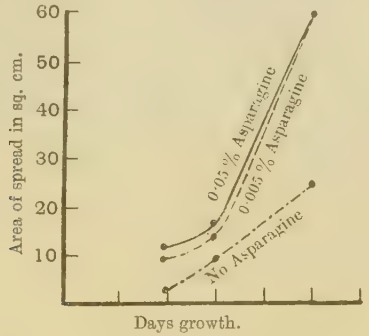

Fig. 10. *B. dendroides*. Effect on asparagine content of medium on spreading.

The additional nitrogen in the medium was supplied as KNO_3 and the effect of this compound on the spreading was therefore tested. The medium used in this and the next experiment had the following composition, and to it KNO_3 and asparagine were added in the amounts indicated.

K_2HPO_4 ...	1·0 gm.	$CaCl_2$ ...	0·1 gm.
$MgSO_4.7H_2O$ ...	0·2 ,,	$FeCl_3$ ...	0·002 ,,
NaCl	0·1 ,,	Mannitol ...	1·0 ,,
Agar	15·0 ,,	Water ...	1000 c.c.

Table IV shows the areas of spreading growth of *B. dendroides* after two and eight days incubation at 20° C. on media nitrogen supply as shown.

Table IV.

Effect of Nitrate and Asparagine on the spreading of Bacillus dendroides.

Medium	Source of nitrogen	Area of spread of *B. dendroides* in 2 days sq. cm.	Area of spread of *B. dendroides* in 4 days sq. cm.
A.	0·05 % asparagine	3·3	56·5
B.	0·05 % asparagine + 0·05 % KNO_3	2·8	58·6
C.	0·05 % KNO_3	0·73	12·6
D.	0·1 % KNO_3	0·58	13·4

It will be seen that complete omission of KNO_3 in medium A is without effect on the spreading, though the omission of asparagine checks the spreading even where the KNO_3 is increased to 0·1 per cent. A reduction in the KNO_3 content below 0·05 per cent. is therefore of no assistance in reducing "spreading."

The effect of a higher concentration of KNO_3 was next tried. Two media were compared, having nitrogen supplied as follows:

A. 0·2 per cent. KNO_3 0·05 per cent. asparagine.

B. 0·05 per cent. KNO_3 0·05 per cent. asparagine.

Fig. 11 shows the increase in area of surface growth of *B. dendroides* on these two media, each point on the curve representing the mean of twenty parallel plates.

It will be seen that in the presence of asparagine, the higher concentration of KNO_3 increases the spreading on platings. A suspension of Barnfield soil was plated on the above media, and on one containing 0·1 per cent. KNO_3 + 0·05 per cent. asparagine, and no significant difference could be found in the number of colonies developing on the three media. There is thus no advantage in lowering the concentration of KNO_3 below 0·05 per cent. while a higher concentration tends to stimulate spreading. This percentage was therefore adopted for use in the medium.

The additional source of energy in the medium was supplied as mannitol. This compound was used in preference to a sugar for reasons, dealt with below, connected with the change in reaction during sterilisation. Experiments on the effect of varying percentages of mannitol on the spreading of *B. dendroides* were made. It was found that an increase in the mannitol content from 0·05 per cent. to 0·1 per cent. did not stimulate spreading but that a further increase of 0·2 per cent. caused slightly more spreading growth. Counts were also made of the number of colonies

which developed from a single diluted suspension of Rothamsted soil on media containing 0·05 per cent., 0·1 per cent. and 0·2 per cent. mannitol. Eight parallel platings of each medium were poured and incubated for seven days at 20° C. No significant differences in the number of colonies, on the different media, were found. It was decided to include 0·1 per cent. mannitol in the medium as this gives the maximum energy supply without increasing "spreading."

Attention was also turned to the effect of phosphate supply on the spreading of *B. dendroides*. The increase in area of surface growth of this organism was measured on media containing 0·2 per cent., 0·1 per cent., 0·05 per cent. and 0·025 per cent. K_2HPO_4 respectively. Variation of the concentration of K_2HPO_4 between these limits was found to be without significant influence on the "spreading."

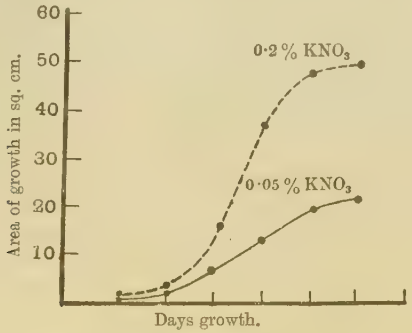

Fig. 11. *B. dendroides*. Effect of nitrate content of medium on spreading.

3. Maintenance of Standard Reaction.

One of the features necessary in a medium to be used in quantitative work is that its reaction should not vary sufficiently to affect the colony development.

The reaction is commonly standardised just before sterilisation. It is during sterilisation that changes occur which are not always constant, so that uniformity in the sterile medium may be lost. In developing a medium, it is therefore important to consider the change in hydrogen ion concentration which will occur during sterilisation. If possible, the medium should be so constituted that this change shall not be sufficient to affect colony development. In this portion of the work, therefore, it was necessary, firstly, to ascertain the limits of change in reaction which might occur in the medium without affecting the number of colonies that

developed, and secondly, to develop a medium whose change of reaction during sterilisation would not reach this limit.

In the measurements of H-ion concentration involved in this work, the indicator method developed by Gillespie(12) was used. This method depends on the assumption that, at any given H-ion concentration, a definite percentage of the indicator is in the acid form and the remainder in the alkaline form. If it is known what these proportions are for a given indicator at a given reaction, the colour shown by the indicator in a solution of this reaction, can be imitated by dividing the indicator in the correct proportions between two solutions, one of which contains excess of acid and the other excess of alkali. These ratios have been ascertained by Gillespie for a number of indicators over a range of *p*H values. Colour standards prepared by this method consist of pairs of tubes, one containing dilute acid and the other dilute alkali. Each pair together contains ten drops of the indicator, these drops being divided between the two tubes according to the ratio ascertained for the *p*H value required.

Before employing this technique, it was thought advisable to test the accuracy of readings obtained with it, as compared with the method of Clark and Lubs(13) in which the indicator colour standards are made up in standard buffer solutions of definite *p*H value. These tests of Gillespie's method were carried out at Rothamsted by Mr E. A. Fisher, to whom, also, I am indebted for much help and advice throughout the work connected with the reaction of the medium. The following indicators were tested:

	Range
Brom Cresol Purple (Dibromo-o-cresolsulphonphthalein)	*p*H 5·6–*p*H 6·8
Brom Thymol Blue (Dibromo-thymolsulphonphthalein)	*p*H 6·5–*p*H 7·7
Phenol Red (Phenolsulphonphthalein)	*p*H 7·2–*p*H 8·3

With each indicator, seven pairs of tubes were made up as colour standards, as described by Gillespie. The indicators were added in drop ratios ranging from 8 acid:2 alkaline, to 2 acid:8 alkaline. The colour of each pair of tubes was compared with a series of colour standard tubes prepared by Clark and Lubs' method in each of which ten drops of indicator were added to 10 c.c. of a buffer solution of known H-ion concentration.

The readings thus obtained are shown in Fig. 12, in which the actual readings are plotted on "smoothed" curves. In those cases where the actual readings lay off the smoothed curves, the pairs of drop ratio tubes were made up several times and invariably gave similar readings. It is therefore believed that these irregularities were due to slight errors in

the buffer solution standards. Where they differ from ours the "smoothed" curve readings, given by Gillespie, are plotted in broken line beside our readings. It will be seen that the two series agree closely in the case of Brom Thymol Blue, but that there is a constant disagreement of about 0·05 *p*H on the alkaline side in the case of Phenol Red and of about

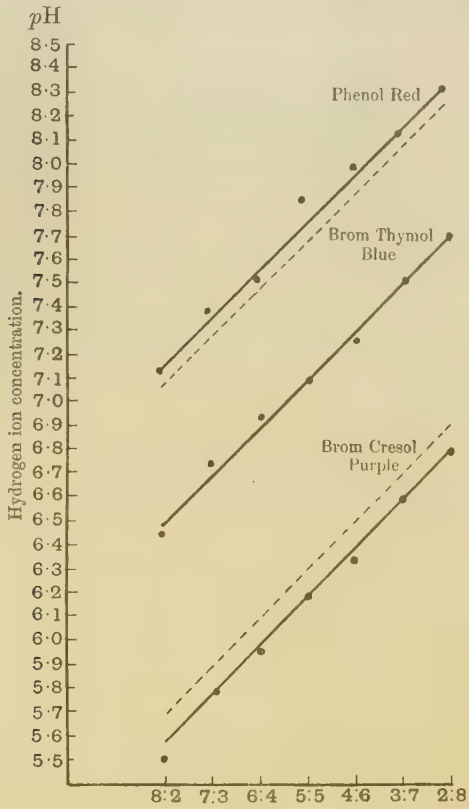

Fig. 12. Drop ratios. (Acid indicator: alkaline indicator.)

0·1 *p*H on the acid side, with Brom Cresol Purple. It is considered probable that this is due to slight differences in the samples of indicator used in the two cases.

The smoothed curves thus experimentally obtained were used in making the readings of *p*H value in the work described below.

In investigating the effect of the H-ion concentration of the substratum on the number of colonies that developed thereon a medium of the following composition was employed:

K_2HPO_4	...	1·0 gm.	KNO_3	...	0·5 gm.
$MgSO_4.7H_2O$	...	0·2 ,,	Asparagine	...	0·5 ,,
NaCl	...	0·1 ,,	Mannitol	...	1·0 ,,
$CaCl_2$	...	0·1 ,,	Agar	...	1·5 ,,
$FeCl_3$	...	0·002 ,,	Distilled water		1000 c.c.

The medium was filtered at 100° C. and divided into 200 c.c. portions which were sterilised at 15 lbs. pressure for 15 minutes in the autoclave.

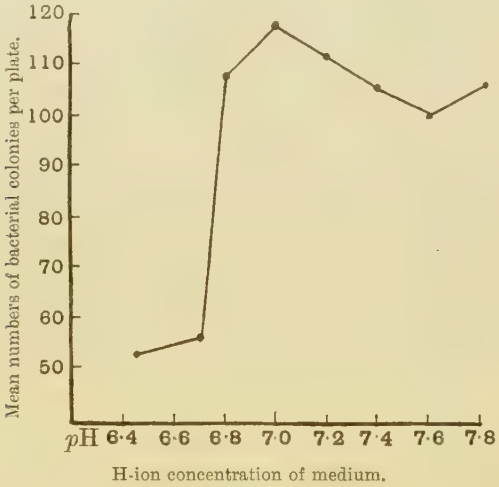

Fig. 13. Effect of the reaction of the medium on colony development.

The H-ion concentrations of the media were then adjusted to values ranging from *p*H 6·45 to *p*H 7·8, with sterile *N*/10 HCl and *N*/10 NaOH, using aseptic technique. A single diluted suspension of Rothamsted soil was plated on each medium, six to eight parallel platings being made in each case. The colonies were counted after 10 days incubation at 20° C. In Fig. 13 the mean number of colonies per plate on each medium is plotted against the *p*H value. The differences in colony development on media ranging from *p*H 6·8 to *p*H 7·8 are barely significant, having regard to the variance between parallel platings, there being, however, some indication of an optimum reaction near neutrality. On the acid side,

however, there is a remarkable fall in colony development on media having an acidity higher than *p*H 6·8.

Thus, in preparing a standard medium, some latitude for changes in reaction during sterilisation is permissible, if its reaction be kept within a range of from *p*H 7 to *p*H 7·8. On the other hand, if the medium be on the acid side of neutrality, a slight increase in its acidity may cause its H-ion concentration to reach the critical point involving a marked fall in colony development.

For this reason, the use of an ammonium salt in a standard medium is a disadvantage, for if such a medium be brought to the alkaline side of neutrality with NaOH, some of the ammonia is liberated, and, during sterilisation, is driven off, bringing the reaction back to neutral point. A slight hydrolysis of the carbohydrate constituent of the medium during sterilisation is now sufficient to bring the H-ion concentration up to a dangerous point. Thus if Conn's sodium asparaginate agar(3) (containing ammonium phosphate) be adjusted to a slightly alkaline reaction before sterilisation, the H-ion concentration of the medium after autoclaving is found to be approximately *p*H 6·8, so that a slight further increase in acidity, arising from any cause, would harmfully affect the medium.

From these considerations, it was decided to use nitrate as the inorganic nitrogen source in the medium. Trials showed that the colony development was as good on a neutral medium containing nitrate as on one containing an ammonium salt, while, in the former case, the risk of a detrimental increase in acidity, during autoclaving, need not be incurred. The following comparison shows the advantage of nitrate in this connection. The medium CV (see p. 259) was made up with 0·05 per cent. asparagine, and divided into two portions to one of which 0·1 per cent. $(NH_4)_2SO_4$ was added, and to the other equivalent nitrogen in the form of KNO_3. The reaction of both media was standardised to *p*H 7·4 and the media were autoclaved at 15 lbs. pressure for 15 minutes. After sterilisation the reaction of the ammonium sulphate medium was found to be *p*H 6·7, while that of the nitrate medium was *p*H 7·2.

The percentage of KNO_3 used in the count medium was finally fixed at 0·05 per cent. as a result of work above described on the control of spreading colonies.

The chief cause of the development of acidity in media during sterilisation is believed to be the hydrolysis of the carbohydrate constituent. I therefore made tests of media containing various sugars and related compounds to discover which source of energy material was most suitable.

The following medium was used as a basis in these tests:

K_2HPO_4 ...	1·0 gm.	KNO_3 ...	0·5 gm.
$MgSO_4.7H_2O$ ...	0·2 ,,	Asparagine	0·5 ,,
CaCl	0·1 ,,	Agar ...	15·0 ,,
NaCl	0·1 ,,	Water ...	1000 c.c.
$FeCl_3$	0·002 ,,		

This medium was divided into five portions to which were added the following compounds in 0·1 per cent. concentration:

A. Dextrose. B. Saccharose. C. Mannitol. D. Lactose. E. Glycerine.

The media were adjusted to a H-ion concentration of *p*H 7·05 immediately before sterilisation for 15 minutes at 15 lbs. pressure. Directly after autoclaving, the reaction was again measured. Six parallel platings of a diluted suspension of Rothamsted soil were made on each medium. Table V shows the changes of reaction during sterilisation and the mean number of colonies per plate with each medium. Table VI shows the results of another similar experiment in which glucose, saccharose and mannitol media were compared.

Table V.

Change in Reaction of Media on Sterilisation.

Energy material	*p*H value before sterilisation adjusted to	*p*H value after sterilisation	No. of colonies on each plate	Mean no. of colonies
Dextrose	7·05	6·7	13, 10, 13, 18, 15, 14	13·8
Saccharose	7·05	6·8	19, 18, 16, 11, 10, 10	14·0
Mannitol	7·05	6·9	19, 18, 17, 16, 13, 10	15·5
Lactose	7·05	6·7	13, 12, 12, 11, 10, 10	11·3
Glycerine	7·05	6·75	18, 13, 12, Sp. Sp. Sp.	14·3

Table VI.

Change in Reaction of Media on Sterilisation.

Energy material	*p*H value before sterilisation	*p*H value after sterilisation	No. of colonies on each plate	Mean no. of colonies
Dextrose	7·2	6·7	14, 21, 13, 14, 14, 13, 14, 13	14·5
Saccharose	7·2	6·7	19, 17, 17, 16, 15, 14, 13	15·8
Mannitol	7·2	6·95	19, 19, 22, 22, 18, 17, 17, 17	18·9

It will be seen that the medium containing mannitol changes least in reaction during sterilisation and at the same time gives a good colony development. With lactose, the number of colonies was somewhat reduced and the individual colonies were dwarfed. With glycerine, marked

spreading of *B. dendroides* occurred on the plates. Mannitol was consequently adopted as the energy source, in addition to the asparagine, in the count medium, and subsequent work has shown its advantage, both on account of the slight change in reaction produced by it on autoclaving, and of the good development of colonies on the medium.

4. Preparation of the Medium.

On account of the changes and interactions which take place in a nutrient medium in the course of its preparation, it is necessary, in order to obtain uniform results with it, that the method of preparation should be carefully standardised. The need for this precaution is well shown by the effect of variations in the method of filtration, discussed below.

The medium here developed has the following composition:

K_2HPO_4 ...	1·0 gm.	KNO_3 ...	0·5 gm.
$MgSO_4 + 7H_2O$	0·2 ,,	Asparagine	0·5 ,,
$CaCl_2$	0·1 ,,	Mannitol ...	1·0 ,,
NaCl	0·1 ,,	Agar ...	15·0 ,,
$FeCl_3$	0·002 ,,	Water to	1000 c.c.

In making up this medium, the following technique was finally adopted. The phosphate, nitrate and asparagine are dissolved in the distilled water and the $MgSO_4$, $CaCl_2$, NaCl and $FeCl_3$ added from standard solutions, in the order named. The agar is then added and dissolved at 100° C. The medium is then filtered at this temperature, by being passed twice through a layer of absorbent cotton-wool half an inch thick. The mannitol is then dissolved in the filtrate. It is then allowed to cool to 60° C. and its reaction adjusted against Brom Thymol Blue to *p*H 7·4. The medium is then poured into tubes and sterilised at 15 lbs. pressure for 15 minutes.

In the earliest work done with this medium, some differences were found between different batches of medium. These differences were eventually traced to variations in the temperature at which filtration was carried out. The effect of the temperature of filtration is shown in the following experiment. Three litres of the medium were made up and divided into two portions, one of which (A) was filtered through cotton-wool at 100° C. and the other (B) at 50° C. A single diluted suspension of Rothamsted soil was plated on the two media and the mean number of colonies on ten parallel platings on each medium was taken. The results are shown in Table VII, and indicate a perceptible fall in the nutritive value of the medium when filtered at the lower temperature. (See also Fisher, Thornton and Mackenzie(2).)

Table VII.

	Medium filtered at 100° C.	Medium filtered at 50° C.
Colonies on each plate	68	49
	65	49
	65	48
	58	48
	57	47
	54	45
	53	44
	53	41
	51	39
	41	39
Mean	56·5	44·9

Tests were also made as to the comparative advantages of filtration through filter paper and cotton-wool. No advantage was found in the former method, either with reference to the total number of colonies developing, or to the uniformity between batches of medium separately filtered.

Length and Temperature of Incubation. In working with this medium, the best results have been obtained by incubating the platings at 20° C. for 10 to 12 days. In a shorter period the slow-growing colonies have either not developed or are very small. These results agree with the finding of Cunningham (14).

5. Tests of the Count Medium.

There are two respects in which a medium for use in quantitative work should display uniformity. In the first place, it must be reproducible, that is, different batches of medium should be similar in the results obtained with them. Secondly, parallel platings of a suspension of soil, made with a single batch of medium, should develop the same number of colonies within the limits of random sampling variance. Uniformity in this latter respect will depend mainly upon limitation of the growth of fast growing organisms and especially of moulds and bacteria that form spreading colonies or develop toxic products, whose chance appearance on platings may affect the number of colonies developing thereon. These two aspects of the medium must be separately tested.

The capacity for colony development on the present medium has been found to be closely reproducible in different batches, if the method of preparation be carefully standardised. In the following test, five batches of medium were separately prepared, and a single suspension of

Rothamsted soil plated on them, eight parallel platings being made of each medium. Table VIII shows the colonies developing on each plate. It will be seen that no significant differences are shown between the different batches.

Table VIII.

Colony Development from a Single Soil Suspension, Plated on five different Batches of Count Medium.

	Medium A	Medium B	Medium C	Medium D	Medium E
Number of colonies on each plate	92	88	88	82	85
	84	85	88	82	84
	80	78	82	80	80
	77	78	80	76	76
	76	76	78	74	73
	75	75	76	70	70
	75	75	75	70	67
	72	70	75	68	Sp.
Mean no. colonies per plate	78·87	78·12	80·25	75·25	76·43

The uniformity between parallel platings on the same batch of medium has been studied from about 3000 platings made in the Protozoology and Bacteriology Departments at Rothamsted. A statistical analysis of this mass of data has been made by Mr R. A. Fisher (Fisher, Thornton and Mackenzie(2)). The results, which are on the whole quite satisfactory, are published separately.

About 4000 platings have been made on the medium since its development. In the majority Rothamsted soil was used, but 240 of the platings were of a light ironstone soil from Kingsthorpe, Northamptonshire. Although "spreading" organisms occur on about 40 per cent. of the platings of Rothamsted soil, only some 3 per cent. of the platings were lost owing to the development of spreading colonies over the surface.

6. Summary.

1. For bacterial count work the first essential in a medium is that it should be uniform and reproducible in its results.

2. In the medium here described, details of which are given on p. 271, reproducibility has been achieved by the use of pure chemical compounds in an agar medium and by selection of such constituents as will not produce a significant change of reaction during sterilisation.

3. On agar media, surface spreading colonies interfere with the accuracy of the results. A special study was made of a common spreading

organism, *B. dendroides*. This organism spreads by active motility, and the factors controlling its spread were found to be (1) the existence of a surface film of water on the agar, and (2) the rate of multiplication previous to the drying of this film. A medium was developed on which this rate of multiplication was greatly reduced and on which, consequently, spreading is greatly restricted.

4. Tests of the medium have shown that the results obtained with it are uniform and can be reproduced in different batches of medium.

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STUDIES ON THE APPLE CANKER FUNGUS

II. CANKER INFECTION OF APPLE TREES THROUGH SCAB WOUNDS[1]

By S. P. WILTSHIRE, B.A., B.Sc.

(*University of Bristol Agricultural and Horticultural Research Station, Long Ashton.*)

(With Plate XII.)

Introduction.

In a previous paper(1) reference was made to the fact that the canker fungus *Nectria galligena*, Bres., can enter the apple tree through the wounds caused by the scab fungus *Venturia inaequalis*. It is the purpose of this paper to describe this process in detail.

Symptoms.

The scab fungus infects the shoots of susceptible varieties of apples during the autumn and winter following their growth, the first infections usually being found before the trees defoliate. In the spring most of the pustules are surrounded by a cork layer and are subsequently completely excluded from the tree, the only trace of the infection finally being a slight roughness of the bark.

Sometimes, however, this course of events is disturbed. The cortex round the small scab pustule shows signs of blackening, and this is accompanied in some cases by a swelling of the bark due to the growth of the tissues beneath the infection (see Pl. XII, fig. 1). Very early stages, in which the discoloration is extremely slight, can sometimes be identified. When the canker fungus has once got in (for as will be seen later this difference from the normal development is due to *Nectria galligena*, Bres.) it usually develops so rapidly that an area about 5 mm. in diameter is completely killed and blackened before any attempt at phellogen formation becomes effective. The canker area is usually somewhat sunken, there is no crack in the bark between the healthy

[1] A grant in aid of publication has been made for this communication.

and diseased tissue and the little scab infection can often be identified in the middle of the scar. In the autumn and winter canker infections of scab wounds are thus most frequently found in this stage (see Fig. 2). Later stages of development are often characterised by the formation of well-defined cracks at the edge of the infected area and a slight swelling of the adjacent tissue (see Figs. 3 and 4). If the tree is sufficiently vigorous to form a cork layer round such a scar before the wood has become infected, the canker makes very little progress, and the tree makes a good fight against the fungus. Often, however, the whole of the cortex becomes infected and the fungus reaches the woody tissues. In these cases, examples of which are seen in Figs. 5 and 6, the scar is more like a normal canker produced by the canker fungus. It is somewhat difficult to assign any particular method of infection to a mature canker. The presence of the concentric cracks in the bark, however, localises the original point of infection and the appearance of this spot is sometimes strongly suggestive of infection through a scab wound. Fig. 7 is a photograph of such a case and other instances have been found on the pear as well as the apple. Fructifications of the fungus are not borne until the canker is well developed, but on keeping young infections in a moist chamber for two or three days, a few small conidial pustules generally appear and afford evidence of the presence of the canker fungus.

The occurrence of this type of infection has not been found to be nearly so common as that of the leaf scar infection previously described but it is probably as prevalent as the infection which takes place through woolly aphis galls. In some years when the autumn has been specially damp, the shoots of the previous winter are often found to be killed off in large numbers. Such shoots are usually heavily infected with scab and although they frequently bear leaf scar infections of the canker fungus, it is probable that canker infections of scab wounds are responsible for a good proportion of the damage.

Microscopic Details.

The establishment of the canker mycelium upon the scab stroma. Early in the autumn, when the scab pustules are very small and before any really definite signs of infection can be observed by the naked eye, microscopical examination has revealed the presence of *Nectria galligena* in a number of instances. The minute cracks which occur in the bark covering the young scab infection afford a favourable lodgment for the conidia of the canker fungus, which germinate readily under the moist

conditions usually prevailing in the autumn. The mycelium thus produced proceeds to establish itself on the stroma of the scab fungus. This done, it begins to form typical conidia, which afford proof of the identity of the canker fungus. Fig. 8 shows a drawing of a small pustule of the canker fungus growing just over a young scab infection. The material concerned was freshly collected from the plantation and the conidia illustrated had developed there under natural conditions. A section through a young scab infection attacked by *Nectria galligena* which has reached a slightly later stage is shown in Fig. 9, the central portion of which is enlarged in Fig. 10. Here the scab fungus has burst open the bark and the canker fungus has attacked the mycelium of *Venturia inaequalis* and produced numerous conidia. It is rather difficult to distinguish between the mycelia of the two fungi, but generally speaking in a mixed stroma, the mycelium of the scab fungus appears dark and somewhat thick-walled whilst that of the canker fungus is hyaline, less distinct and not so robust. Its cells, too, are rather smaller in size than those of the scab fungus. The two mycelia are not absolutely intermingled but are naturally divided into areas. In Fig. 10 the areas (*a*) and (*b*) are probably *Nectria* mycelium whilst the areas (*c*) and (*d*) are probably scab.

The penetration of Nectria galligena *from the scab stroma into the cortex.* As soon as the canker fungus has gained a firm hold on a scab pustule, a struggle between host and parasite commences. It has been shown (2) that if a cut is made in the cortex of an apple tree in such a way that it does not extend to the wood, and if conidia or hyphae of *Nectria galligena* are placed on such a wound, the host merely forms a cork layer round the portion of cortex which becomes infected and the fungus fails to establish itself. The experiments were chiefly carried out in the summer when the trees were active, and the results were so positive that for some time it appeared very difficult to understand how the canker fungus could infect from a shallow injury such as that effected by the scab fungus. It is indeed probable that, in some cases at any rate, such a cork layer is effective in preventing the development of canker even in cases of *Nectria* infection of scab pustules. In Fig. 9 the whole of the infected portion is surrounded by a cork layer formed some distance below the original seat of the trouble. If such a layer became matured before the *Nectria* reached it, then it is extremely probable that the latter could progress no further, unless assisted by a fresh development of the scab fungus.

With the formation of a phellogen, the cortical cells below frequently begin to divide to produce new tissue in addition to that arising from

the activity of the phellogen. The result of this growth is that cracks sometimes occur in the bark in the vicinity of the infected portion (see Fig. 9); but although mycelium is sometimes found in such places, its occurrence is not frequent enough to suggest that infection by *Nectria* is secured by this means.

The cork layer, however, is not always developed quickly enough to confine the canker fungus to the outside of the barrier. The scab fungus is able to penetrate suberised tissue and its normal procedure is simply to grow through any cork layer formed below it, especially at the edges of the infected region. *Nectria* appears to follow the *Venturia* to some extent. The antagonism which might be expected to be exerted by the scab fungus appears to be quickly overcome and *Nectria* subsequently dominates the situation. Its hyphae begin to grow inwards between the cells of the cortical tissue, which towards the outside of the stem, includes very few intercellular spaces. The penetration of the cortical cells, however, is not general, but confined at first to a few strands of mycelium, which appear to be formed in the following manner. One hypha or a strand of a few hyphae pushes its way somewhat deeply into the tissue, travelling almost invariably through the middle lamellae. Other hyphae follow pushing their way alongside the original hypha which of course continues its growth. In this way a whole strand of mycelium is built up consisting of 20–30 or even more hyphae. Several such strands can frequently be found radiating out from an infected scab pustule. In Fig. 11 an excellent example of a young hyphal strand (*A*) penetrating from the subepidermal stroma (*C*) can be seen; in the same figure a more fully developed strand of many hyphae can be recognised at (*B*). In Fig 9 also it is possible to follow the subepidermal mycelium along from the place of the original scab infection towards the left, where a strand of mycelium is seen penetrating inwards towards the wood. These strands of mycelium are very characteristic of this type of *Nectria* infection. If the cork layer is not in an advanced stage of development, the *Nectria* hyphae are capable of penetrating between its cells through the middle lamellae of the cell walls. Fig. 12, which is a photograph of the section adjacent to that of Fig. 11 in the same series, shows such a stage. At (*D*) the mycelial strand consisting only of very few hyphae is penetrating the immature cork layer. The hyphae could be traced back to a much larger strand which is slightly out of focus in the photograph but which can be seen at (*B*) and which originated from the subepidermal mycelium (*C*). The young strand (*A*) (corresponding to (*A*) in Fig. 11) which has not yet reached the phellogen can just be distinguished. Once

the mycelium has penetrated beyond the cork layer, the host sometimes makes a half-hearted attempt to stop its progress by the formation of a second phellogen as shown in Fig. 13. The *Nectria*, however, is powerful enough to penetrate the new phellogen in the same way as it did the old one, provided that cork formation has not yet taken place.

From the appearance of sections of old infections, the fungus seems to be capable of secreting some substance, probably of the nature of an enzyme, which is able to attack the cell walls of the cortical tissue. These are not totally destroyed but they lose their power of staining with Fuchsin and the tissue so affected appears indistinct and disorganised. Whether the secretion acts in advance of the *Nectria* mycelium is rather difficult to determine exactly but it appears to be probable, for cells on the outside of the infected area frequently have their contents coagulated and the cell walls rather heavily stained, before mycelium can be found among them. This stage, the first step in the disintegration of the tissue, is followed by the loss of the staining powers and by the advance of the mycelium in the middle lamellae between the cells in the intercellular spaces and in the cells themselves. The nature of the secretory substance has not been investigated. If present, it probably begins to take effect from the very early stages of infection and is perhaps a potent factor in overcoming the resistance of the host.

In this connection, it might be well to refer to some infection experiments carried out some years ago. In these it was sometimes found that if *Nectria* conidia were placed on superficial wounds on cut shoots under a bell-jar, the fungus penetrated to the cortex even when no intercellular spaces were exposed and before any cork layer was developed. The delay in forming a wound cork was considered to be due to the dormant and unhealthy condition of the host, as most of the experiments were made in winter and all of them on cut shoots which could not be expected to have the same vigour as the living tree. Normally *Nectria* mycelium grows in the intercellular spaces of the cortex and as these do not extend to the outside layers of the cortex except at lenticels it was obvious that in infecting through superficial cuts not over lenticels the fungus would have to pass through the cell tissue. The way this was brought about was by the solution of the middle lamellae of the cell walls. Repetition of the experiments during the summer, on growing trees, invariably resulted in a cork layer being formed round the infected portion and the latter completely excluded as mentioned above. It seems clear, therefore, that the secretions of the canker fungus cannot

pass a well-formed cork layer, but if no such barrier exists or if it is only partially developed then the fungus can progress, apparently by the help of its secretions.

The later stages of infection. Once the canker fungus has effected an entrance to the cortex, it proceeds to grow very rapidly in all directions chiefly in the intercellular spaces. Concurrently with the gradual progress of the infection by the fungus the healthy cortical tissue usually becomes very active, its cells dividing rapidly and the intercellular spaces being more or less obliterated. This rapidly dividing tissue unless protected by a cork layer soon becomes infected with the canker mycelium, and undergoes changes described above. The host plant persists in its efforts to form a wound cork layer especially in the region between the sclerenchymatous bundles of the cortex, apparently to prevent the fungus from entering the wood. Sometimes the growth stimulus of the cells of the developing phellogen layer is so strong that the cells hypertrophy and the whole tissue becomes ruptured at this region. When the infection has penetrated too deeply to be excluded by a cork layer and cannot be prevented from reaching the wood, wound wood is formed, consisting of medullary ray-like cells, and these offer considerably more resistance to the path of the fungus than the vessels, since the contents of the brick-shaped cells of the wound wood become choked up with gummy material which especially collects at the pits through which the hyphae of *Nectria* normally pass. Sometimes the fungus reaches the wood before any wound wood can be formed and in this case wound wood is cut off from the cambium which still remains living round the infected area. In the cortex a strong cork layer is usually formed ultimately round the infected tissue and this has the effect of limiting the infection and is largely responsible for the concentric cracks in the canker scar which are so characteristic of the disease. Not infrequently the stem becomes completely girdled and the whole of the shoot above is killed off.

This manner of infection appears to be unusual amongst fungal parasites. Fungi parasitic on other fungi are known, but for one, unable to penetrate uninjured bark itself, to take advantage of the injury effected by another and subsequently supersede the latter is unique.

Control.

The obvious way of controlling the infection of scab wounds by *Nectria* is to control the autumn infection of the scab fungus on the wood. It is the usual practice to spray against scab in the spring to protect the fruit and no measures beyond cutting out diseased wood are taken

Fig. 1

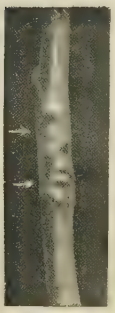

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

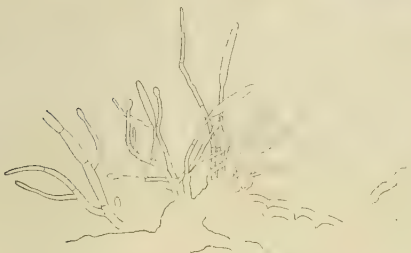

Fig. 8

Fig. 9

Fig. 10

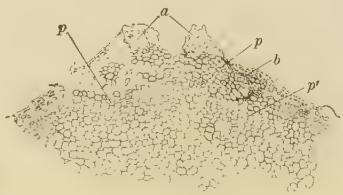

Fig. 13

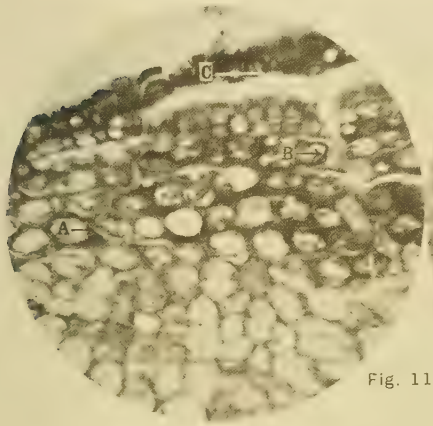

Fig. 11

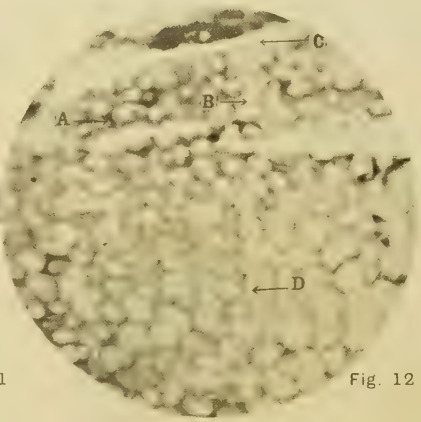

Fig. 12

against the autumn infection. Trials may show that winter spraying immediately after defoliation is effective.

Summary.

The infection of apple trees by the canker fungus through scab infections is described.

The conidia alighting on the exposed scab stroma give rise to a mycelium which attacks the latter and then grows out into the cortex. The fungus is able to pass through any immature cork layer and finally reach the wood.

EXPLANATION OF PLATE XII.

Fig. 1. Young stage in the canker infection of a scab wound. Variety, Lord Suffield. Jan. 16, 1922. ×1·5.

Fig. 2. Similar infections to Fig. 1, but slightly more advanced. Variety, Ecklinville. ×0·7.

Fig. 3. As Fig. 2, but further developed. Variety, Lord Suffield. Jan. 16, 1922. ×1·8.

Fig. 4. As Fig. 3. Variety, Lord Suffield. Jan. 16, 1922. Note the original scab from which the infection started. ×1·8.

Fig. 5. A very active infection on a vigorous shoot of Lord Suffield. Jan. 14, 1922. ×1·5.

Fig. 6. Very active canker in late stage of development. Variety, Lord Suffield. Jan. 16, 1922. ×1·5.

Fig. 7. Mature canker infection of scab wound. March 3, 1922. ×0·8.

Fig. 8. Camera lucida drawing of young canker pustule on outside of a scab infection. Variety, King of the Pippins. Nov. 1921. ×420.

Fig. 9. A trans. section through a young infection of a scab wound by *Nectria galligena*. King of the Pippins. Nov. 1921. ×38.

Fig. 10. Central portion of Fig. 9 enlarged to show the *Nectria* mycelium (*a*, *b*) growing on the *Venturia inaequalis* mycelium (*c*, *d*). Note the characteristic conidia of *Nectria galligena*. ×250.

Fig. 11. Trans. section of an infection showing the formation of hyphal strands of *Nectria galligena*. A young strand can be seen at *A*, and an older one at *B*, both strands being derived from the sub-epidermal mycelia *C*.

Fig. 12. Adjacent section to that of Fig. 11, showing the penetration of the young phellogen at *D*. The other lettering corresponds to that of Fig. 11.

Fig. 13. Trans. section through a canker infection of a scab pustule (*a*) showing the growth of the mycelial strand of *Nectria* (*b*) through the phellogen (*p*) into the cortex. A new phellogen (*p'*) is organised around the advancing mycelium. ×48.

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THE INSECT AND OTHER INVERTEBRATE FAUNA OF ARABLE LAND AT ROTHAMSTED[1]

By HUBERT M. MORRIS, M.Sc., F.E.S.

(*Entomological Dept., Institute of Plant Pathology, Rothamsted Experimental Station, Harpenden.*)

(With 7 Text-figures.)

CONTENTS.

This investigation was carried out from February 1920, to January 1921, with the object of obtaining information as to the species of insects and other invertebrates present in the soil of an arable field. The various species and their relative numbers, the depth at which these organisms occur, and the effect upon them of the application of farmyard manure to the land were the principal points considered.

I am very much indebted to Dr A. D. Imms for suggestions and advice throughout this investigation. I am also indebted to Miss K. Warington for information regarding the weeds; to Mr G. C. Sawyer for estimating the nitrogen content of several groups; to Mr H. J. Page for

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the mechanical and chemical analyses; and for help in the identification of species of *Insecta*, *Myriapoda* and *Arachnida* to Messrs S. G. Brade-Birks, J. M. Brown, E. A. Butler, H. St J. K. Donisthorpe, F. W. Edwards, H. F. Fryer, J. E. Hull, R. C. L. Perkins.

1. Description of the Area examined.

The area dealt with in this investigation was the Broadbalk field belonging to the Rothamsted Experimental Station, Harpenden. The soil of the Rothamsted fields is "clay with flints," which overlies chalk.

Broadbalk field is roughly rectangular in shape, the long sides running W.N.W. to E.S.E., and it lies on a gentle slope, the south-east side being the lowest, this side being slightly over 400 feet above sea level.

The field is divided into a number of plots, of which numbers 2 and 3 were dealt with in this investigation. Plot 2 has received annually a dressing of farmyard manure at the rate of fourteen tons to the acre since 1843. Plot 3, which is a control, has received no farmyard or artificial manure of any kind since the commencement of the experiments in 1843 and actually since 1839.

These plots are about half an acre in area, and lie side by side along the northern side of the field, being separated by a path two yards wide.

The effect of the different treatment of the plots is very noticeable in their yield of grain and straw, and in the general growth of the wheat and weeds. This treatment having been the same in either case for so many years makes them particularly well fitted for an investigation of the soil fauna which they support.

The plots were ploughed on October 13th, the manure having been applied to plot 2 just previously.

2. Method of Investigation.

The samples of soil which were examined in the course of this investigation were taken from the western end of the plots, and were taken from the edges of the plots so as to disturb the soil of the plots as little as possible. Successive samples from the same plot were not taken next to each other, nor were any two samples taken nearer together than about a yard.

The method of taking the sample was as follows. Four iron plates were used, two of them twelve inches long by ten inches wide, one twelve inches long by nine inches wide, and one four inches long by nine inches wide. Each plate had an iron bar fastened to it at the top, and each of the three larger plates had two projecting teeth at the bottom. These

teeth and the lower edges of the plates were kept sharpened in order that they might enter the ground more easily (Fig. 1).

The plates were driven into the ground to form a box nine inches square, the smallest plate being on the side towards the outside of the plot (Fig. 2). A hole was then dug in the path, extending about two feet

Fig. 1. Iron plates and trowel used in taking soil samples.

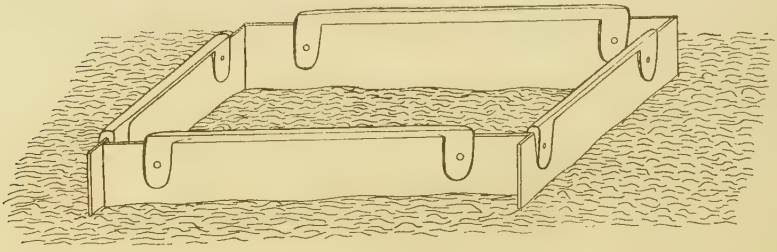


Fig. 2. Plates in position, before any soil has been removed.

from the smallest plate, and about a foot in width, in order to give room to remove the soil from the box. This hole was first made to a depth of about two inches, the front plate was then removed, and by means of the special trowel it was possible to remove the top layer of soil enclosed by the "box." This soil was then extracted to a depth of one inch;

owing to the unevenness of the soil the latter level was measured from the lowest point of the surface. On removal the soil was placed in a linen bag.

The small plate was then replaced and driven down another two inches, and the hole in the path was deepened by about another two inches (Fig. 3). The soil in the box was then removed in the same way as before. The second and succeeding samples were taken at depths of two inches at a time, each being placed in a separate bag.

The soil was removed in this way to a depth of nine inches, giving five samples, which consisted of—I, the soil between the surface and a depth of one inch below the lowest point of the surface; II, the soil

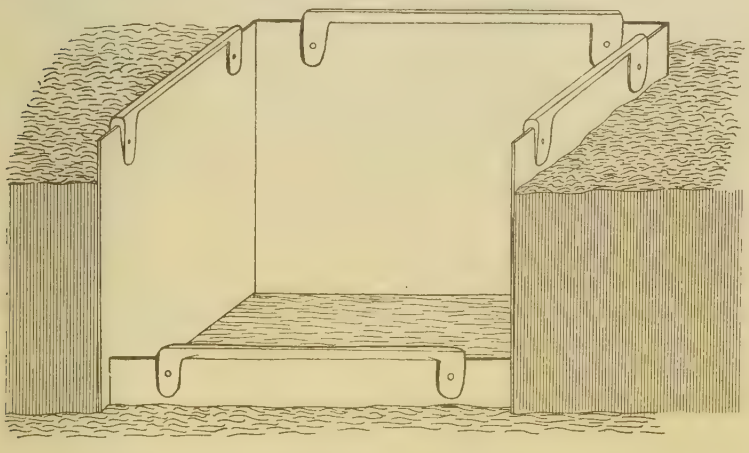


Fig. 3. Plates in position after three samples of soil have been removed.

between a depth of one inch below the lowest point of the surface and a depth of three inches; III, the soil between three inches and five inches; IV, the soil between five inches and seven inches; V, the soil between seven inches and nine inches.

The samples obtained in this way were taken to the laboratory for examination. When the soil was wet it was necessary to spread it out to dry for some time, before it was possible to examine it thoroughly. The examination had to be carried out by crumbling the soil on to sheets of brown paper, and watching for the appearance of insects, etc., as the soil was broken up. The soil was examined over brown paper instead of white, which at first might seem more suitable, because the most

abundant small insects, and the majority of the larvae, were white or light-coloured. Other methods of obtaining the insects, etc., from the soil were considered, but were not found to be feasible. By taking a small quantity of soil at a time, and examining it in this way, it is possible to obtain, probably, practically all the insects, etc., from the soil, although it is likely that a few of the smaller forms would be overlooked.

Twenty-three cubes of soil, each 9″ × 9″ × 9″, were examined in this way, from each plot. They were taken alternately from the plots about every six days, so that a cube was taken from each plot about every 12 days.

The time between successive cubes, however, varied somewhat according to the weather and the condition of the soil. Cubes were not usually taken on rainy days owing to the difficulties entailed in the thorough examination of wet soil.

Since this investigation was completed a method has been devised by means of which the separation of insects and other arthropods from the soil is much facilitated (11).

3. Soil Analyses.

In order to define as exactly as possible the conditions under which the soil fauna was existing on the two plots examined, mechanical and chemical analyses of the soil of both plots were obtained.

Plot 2. Percentages. Moisture (in air-dry soil) 2·22; Nitrogen 0·258; Potash (soluble in HCl) 0·333; Phosphoric acid (soluble in HCl) 0·203; Lime (as $CaCO_3$) 3·43.

Fine gravel 1·63; Coarse sand 2·57; Fine sand 21·96; Silt 17·30; Fine silt I 11·66; Fine silt II 5·06; Clay 13·87; Loss on solution 7·38; Loss on ignition 11·95.

Plot 3. Percentages. Moisture (in air-dry soil) 1·7; Nitrogen 0·114; Potash (soluble in HCl) 0·284; Phosphoric acid (soluble in HCl) 0·099; Lime (as $CaCO_3$) 4·01.

Fine gravel 1·01; Coarse sand 3·17; Fine sand 23·31; Silt 20·36; Fine silt I 6·22; Fine silt II 3·81; Clay 16·56; Loss on solution 6·88; Loss on ignition 8·54.

The figure for loss on ignition includes combined moisture as well as organic matter.

4. Meteorological Conditions.

As the meteorological conditions probably exercise an influence on the soil fauna, especially the rainfall and soil temperature, records of

these were obtained. The soil temperatures were registered by a recording thermometer at a depth of six inches.

These records have been preserved but are not considered in the present instance.

The total rainfall during the period February 1st, 1920, to January 29th, 1921, was 26·459 inches.

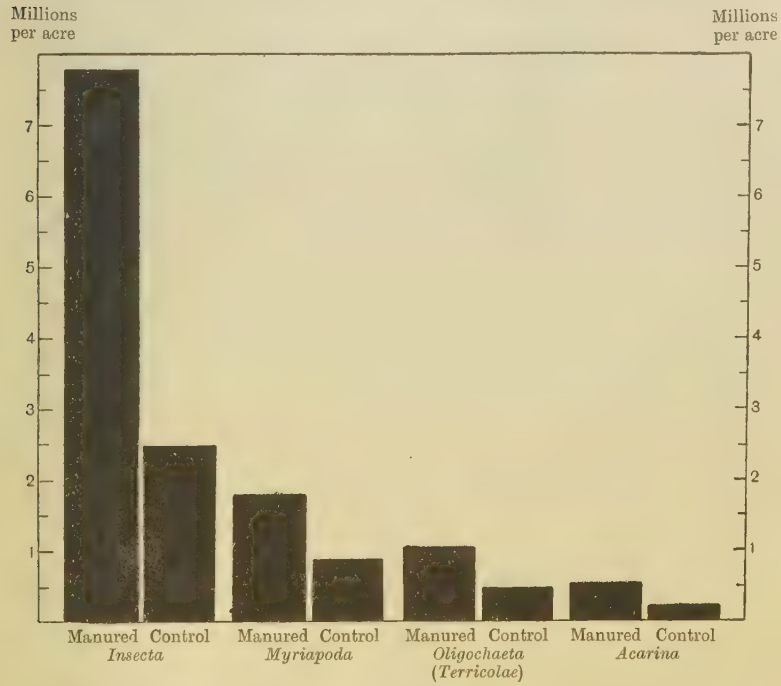


Fig. 4. Number of individuals in the more important groups in the manured and control plots.

5. Occurrence of Weeds.

Plot 2. In the spring the most abundant weeds are *Veronica hederaefolia*, *Scandix pecten* and *Galium aparine*, and in addition to these *Alopecurus agrestis* and *Carduus arvensis* are plentiful.

In the summer *Scandix pecten* and *Galium aparine* are still abundant, and in addition to those occurring earlier, *Caucalis arvensis*, *Equisetum arvense* and *Tussilago farfara* are plentiful, and later still *Convolvulus arvensis* is also prevalent.

Plot 3. In the spring the most plentiful weeds are *Veronica hederaefolia* and *Galium aparine*. In the summer *Tussilago farfara*, *Sonchus arvensis*, *Vicia sativa* and *Lathyrus pratensis* are plentiful and *Alopecurus agrestis*, *Equisetum arvense*, *Carduus arvensis* and *Scabiosa arvensis* are generally distributed, and later still *Convolvulus arvensis* is also plentiful.

6. Soil Fauna of the Manured Plot.

In the following lists the worms have been divided into two groups, those belonging to the sub-order *Terricolae* of the order *Oligochaeta*, which includes the true earthworms, *Lumbricus*, etc., forming one group as *Oligochaeta* (*Terricolae*), and all other worms, probably principally belonging to the family *Enchytraeidae* of the *Oligochaeta*, and to the *Nematoda*, forming the second group as *Oligochaeta* (*Limicolae*), etc.

The numbers following the names have the following meaning—the first numbers give the months during which the species was met with. The first numbers within the brackets give, above, the total number found, and below, in Roman numerals, the levels in which they were found. The second numbers within the brackets give, above, the greatest number found at any one level, and below, in Roman numerals, the level at which they were found. Thus—*Trichocera fuscata* Mg. (larvae) 1–12 $\left(\frac{108}{\text{I–IV}}; \frac{54}{\text{II}}\right)$ indicates that larvae of *Trichocera fuscata* Mg. were found in each month from January to December; 108 were found altogether in samples I, II, III and IV, *i.e.* between the surface and a depth of seven inches, and that 54 of these were found in the second sample, between a depth of one inch and three inches.

The species of insects and other invertebrates present in the manured plot are as follows:

INSECTA.

Collembola. Onychiuridae. *Onychiurus fimetarius* (Linn.) 1–12; *O. ambulans* (Linn.) 1–12; *Tullbergia quadrispina* (Börn.) 2, 9.

Isotomidae. *Isotoma viridis* Bourl. 2–3, 8–10; *I. minor* Schäff. 1, 4, 5, 10; *I. olivacea* (Tullb.) 2; *Folsomia quadrioculata* (Tullb.) 4; *Isotomurus palustris* (Müll.) 10.

Entomobryidae. *Entomobrya multifasciata* (Tullb.) 8; *Lepidocyrtus cyaneus* (Tullb.) 1, 5, 8; *L. albus* Pack. 4; *Orchesella villosa* (Geoff.) 8, 10; *Heteromurus nitidus* Templ. 2, 4, 8, 10, 12.

Smynthuridae. *Smynthurus viridis* (Linn.) 8.

Collembola. All species 1–12 $\left(\frac{710}{\text{I–V}}; \frac{264}{\text{II}}\right)$.

Thysanura. Campodeidae. *Campodea staphylinus* Westw.; *C. gardneri* Bagn.; *C. fragilis* Meinert. Spp. 2–11 $\left(\frac{33}{\text{I–IV}}; \frac{14}{\text{II}}\right)$.

Orthoptera. Forficulidae. *Forficula auricularia* L. 2, 6, 8, 10 $\left(\frac{4}{\text{I–II}}\right)$.

Thysanoptera. Spp. 5–8 $\left(\frac{7}{\text{I}}\right)$.

Hemiptera. CAPSIDAE. *Lygus pastinacae* Fall. 4 $\left(\frac{1}{I}\right)$.

APHIDIDAE. *Aphis* sp. 9 $\left(\frac{1}{I}\right)$.

Lepidoptera. HEPIALIDAE. Unidentified larvae 1–5, 8–12 $\left(\frac{7}{I–V}; \frac{3}{II}\right)$.

Unidentified larvae 3, 8 $\left(\frac{2}{I}\right)$.

Coleoptera. CARABIDAE. *Notiophilus aquaticus* L. 4 $\left(\frac{1}{I}\right)$; *Badister bipustulatus* F. 3 $\left(\frac{1}{I}\right)$; *Bradycellus verbasci* Duft. 8 $\left(\frac{2}{I}\right)$; *Harpalus ruficornis* F. 5 $\left(\frac{1}{I}\right)$; *H. aeneus* F. 2 $\left(\frac{1}{I}\right)$; *Pterostichus madidus* F. 4–6 $\left(\frac{5}{I–III}; \frac{3}{II}\right)$; *Bembidium guttula* F. 2 $\left(\frac{1}{I}\right)$.

HYDROPHYLIDAE. *Helophorus nubilus* F. 5, 6, 8, 9 $\left(\frac{5}{I}\right)$.

STAPHYLINIDAE. *Homalota* spp. 2, 3, 5, 9, 11 $\left(\frac{7}{I–V}\right)$; *Tachyporus hypnorum* F. 5, 8 $\left(\frac{4}{I}\right)$; *Quedius cinctus* Payk. 4, 5 $\left(\frac{2}{I, II}\right)$; *Ocypus morio* Grav. 9 $\left(\frac{1}{II}\right)$; *Philonthus trossulus* Nord. 9 $\left(\frac{1}{I}\right)$; *Lathrobium fulvipenne* Grav. 2 $\left(\frac{1}{IV}\right)$; *L. longulum* Grav. 10 $\left(\frac{1}{V}\right)$; *Scopaeus* sp. 8 $\left(\frac{1}{II}\right)$; *Medon propinquus* Bris. 5 $\left(\frac{1}{I}\right)$; *Stenus subaeneus* Er. 10 $\left(\frac{1}{I}\right)$; *Oxytellus laqueatus* Marsh. 6, 11 $\left(\frac{6}{I–IV}; \frac{2}{I, IV}\right)$; *O. inustus* Grav. 9 $\left(\frac{1}{I}\right)$; *O. sculpturatus* Grav. 4, 6, 9 $\left(\frac{5}{I–IV}; \frac{2}{III}\right)$; *O. nitidulus* Grav. 10 $\left(\frac{1}{III}\right)$; *O. tetracarinatus* Block. 3 $\left(\frac{1}{III}\right)$.

PSELAPHIDAE. *Bryaxis fossulata* Reich. 3 $\left(\frac{1}{I}\right)$.

LATHRIDIIDAE. *Enicmus minutus* L. 10 $\left(\frac{1}{III}\right)$; *Melanophthalma fuscula* Humm. 6 $\left(\frac{1}{I}\right)$.

CUCUJIDAE. *Silvanus surinamensis* L. 6 $\left(\frac{1}{I}\right)$.

ELATERIDAE. *Agriotes sputator* L. 3, 5 $\left(\frac{2}{I}\right)$.

CHRYSOMELIDAE. *Phyllotreta undulata* Kuts. 8 $\left(\frac{1}{I}\right)$; *Plectroscelis concinna* Marsh. 6 $\left(\frac{1}{I}\right)$.

CURCULIONIDAE. *Sitones humeralis* Steph. 8 $\left(\frac{1}{I}\right)$.

LARVAE AND PUPAE—CARABIDAE 1, 4, 5, 9–12 $\left(\frac{14}{I–V}; \frac{9}{II}\right)$; STAPHYLINIDAE 1–6, 10–12 $\left(\frac{103}{I–V}; \frac{36}{I}\right)$; ELATERIDAE 1–12 $\left(\frac{59}{I–V}; \frac{19}{IV}\right)$; TELEPHORIDAE 1, 3, 8, 10 $\left(\frac{4}{I, V}\right)$; CURCULIONIDAE 10, 11 $\left(\frac{2}{III, IV}\right)$; unidentified 4, 12 $\left(\frac{4}{I, II}\right)$.

Diptera. MYCETOPHILIDAE. *Sciara* sp. 12 $\left(\frac{1}{III}\right)$.

CHIRONOMIDAE. *Camptocladius aterrimus* Mg. (Reared from larvae.)

TIPULIDAE. *Pachyrrhina maculosa* Mg. (larva) 4 $\left(\frac{1}{I}\right)$; *P. histrio* F. (larva) 5 $\left(\frac{1}{I}\right)$; *Trichocera fuscata* Mg. (larvae) 1–12 $\left(\frac{108}{I–IV}; \frac{54}{II}\right)$.

SCATOPSIDAE. *Scatopse halterata* Mg. (larvae) 1 $\left(\frac{2}{III, IV}\right)$.

EMPIDAE. Sp. reared from larvae.

Unidentified larvae of the following families also occurred:

CECIDOMYIDAE 1–12 $\left(\frac{58}{I–IV}; \frac{39}{I}\right)$; MYCETOPHILIDAE 1–12 $\left(\frac{35}{I–IV}; \frac{12}{III}\right)$; CHIRONOMIDAE 1–6, 9–12 $\left(\frac{153}{I–III}; \frac{92}{II}\right)$; TIPULIDAE 4, 5, 10, 11 $\left(\frac{5}{I–III}; \frac{4}{I}\right)$; EMPIDAE 1–12 $\left(\frac{48}{I–IV}; \frac{25}{II}\right)$; SYRPHIDAE 1, 4, 9 $\left(\frac{3}{I}\right)$; ANTHOMYIDAE 3, 9–12 $\left(\frac{9}{I–III}; \frac{4}{II, III}\right)$.

Hymenoptera. CHALCIDIDAE. One species, unidentified 6 $\left(\frac{1}{III}\right)$.

FORMICIDAE. *Myrmecina graminicola* Fabr. 2, 8 $\left(\frac{32}{\text{I–V}}; \frac{17}{\text{IV}}\right)$; *Myrmica laevinodis* Nyl. 4–6, 9–10 $\left(\frac{835}{\text{I–V}}; \frac{759}{\text{I}}\right)$; *Acanthomyops* (*Donisthorpea*) *nigra* L. 8 $\left(\frac{8}{\text{I–II}}\right)$.

ANDRENIDAE. *Andrena chrysosceles* Kirby 3 $\left(\frac{1}{\text{III}}\right)$.

"MYRIAPODA[1]."

DIPLOPODA. *Brachydesmus superus mosellanus* Verhoeff 1–12 $\left(\frac{96}{\text{I–V}}; \frac{30}{\text{III}}\right)$; *Cylindroiulus londinensis* var. *caeruleocinctus* (Wood) (=*C. londinensis* var. *teutonicus* (Pocock) of some records) 1–12 $\left(\frac{129}{\text{I–V}}; \frac{41}{\text{II}}\right)$; *Blaniulus guttulatus* (Bosc.) 1–12 $\left(\frac{138}{\text{I–V}}; \frac{34}{\text{V}}\right)$; *Archiboreoiulus pallidus* Brade-Birks 2, 3, 6–12 $\left(\frac{40}{\text{I–V}}; \frac{15}{\text{II}}\right)$.

CHILOPODA. *Lithobius* sp. 8 $\left(\frac{1}{\text{I}}\right)$; *Geophilus longicornis* Leach 1–12 $\left(\frac{57}{\text{I–V}}; \frac{23}{\text{II}}\right)$.

SYMPHYLA. Spp. 1–12 $\left(\frac{64}{\text{I–V}}; \frac{32}{\text{V}}\right)$.

ARACHNIDA.

Areinida. *Porrhomma pygmaeum* Pd. 4 $\left(\frac{1}{\text{I}}\right)$; *P. microphthalmum* Cb. 10 $\left(\frac{1}{\text{IV}}\right)$; *Robertus lividus* Bl. 6 $\left(\frac{1}{\text{III}}\right)$; *Linyphia* spp. 6, 8 $\left(\frac{2}{\text{I, III}}\right)$; *Oedothorax agrestris* Bl. 3 $\left(\frac{1}{\text{I}}\right)$.

Acarina. ANYSTIDAE. *Anystis baccarum* L. 5, 6 $\left(\frac{3}{\text{I}}\right)$.

GAMASIDAE. *Gamasus magnus* Kr. 4–6, 9–12 $\left(\frac{10}{\text{I–III}}; \frac{6}{\text{I}}\right)$; *Gamasus* sp. (immature) 2–5, 8–9 $\left(\frac{13}{\text{I–III}}; \frac{7}{\text{I}}\right)$; *Pergamasus crassipes* L. 4–12 $\left(\frac{11}{\text{I–IV}}; \frac{7}{\text{I}}\right)$; *P. meridionalis* Berl. 10 $\left(\frac{1}{\text{I}}\right)$; *P. hamatus* Koch 3–5, 8–11 $\left(\frac{14}{\text{I–V}}; \frac{5}{\text{II}}\right)$; *P. septrionalis* Oud. 1, 5–12 $\left(\frac{33}{\text{I–III}}; \frac{19}{\text{I}}\right)$; *P. rumiger* Berl. 5, 8, 9 $\left(\frac{5}{\text{I–II}}; \frac{4}{\text{I}}\right)$; *P. alpestris* Berl. 10 $\left(\frac{1}{\text{I}}\right)$; *Pergamasus* spp. (immature) 1–5, 8–10 $\left(\frac{40}{\text{I–IV}}; \frac{17}{\text{I}}\right)$; *Pachylaelaps pectinifer* Berl. 4 $\left(\frac{1}{\text{II}}\right)$.

TARSONEMIDAE. *Pigmephorus morrisii* Hull. 2, 8 $\left(\frac{4}{\text{II, III}}; \frac{3}{\text{III}}\right)$.

TYROGLYPHIDAE. *Rhizoglyphus echinopus* Rob. 3 $\left(\frac{5}{\text{II}}\right)$; *Histiostoma julorum* Koch (hypopus) 3 $\left(\frac{1}{\text{II}}\right)$.

OLIGOCHAETA (*Terricolae*) 1–12 $\left(\frac{300}{\text{I–V}}; \frac{111}{\text{II}}\right)$.

OLIGOCHAETA (*Limicolae*), **NEMATODA**, etc. 1–12 $\left(\frac{1072}{\text{I–V}}; \frac{403}{\text{II}}\right)$.

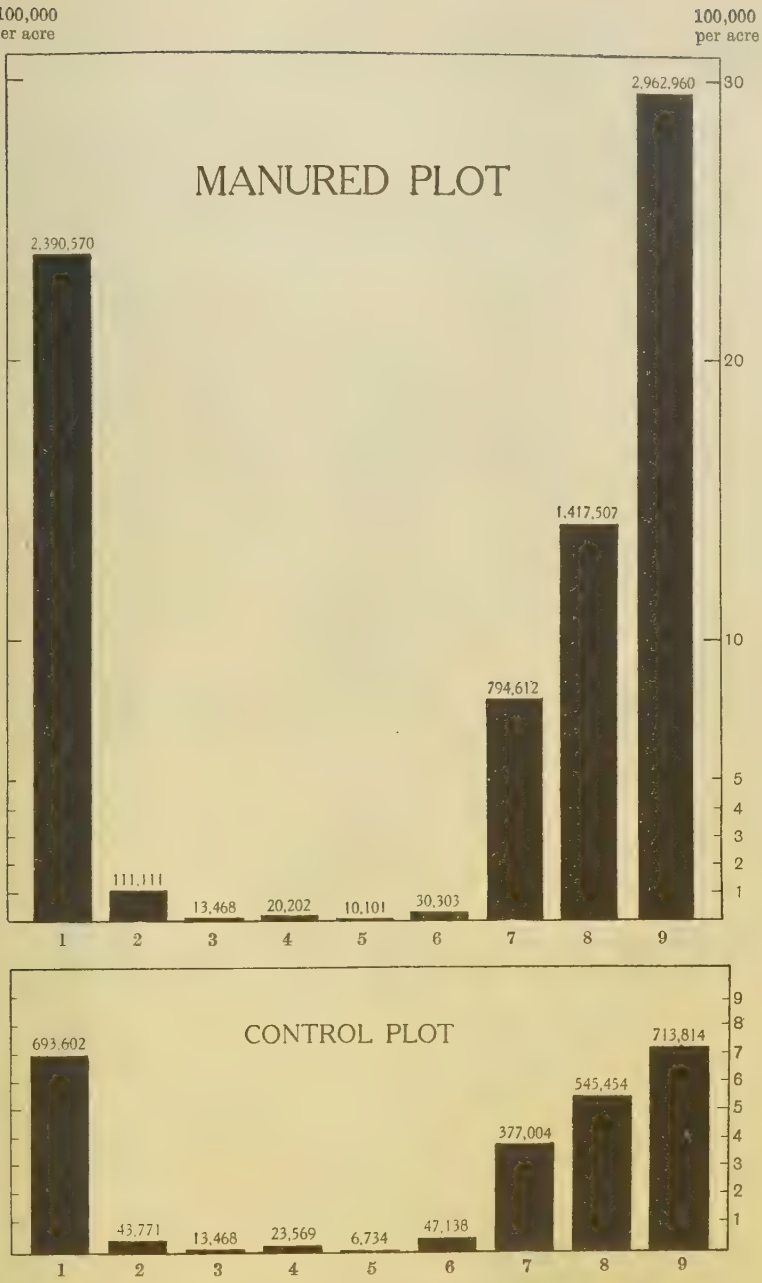
ISOPODA 4, 5, 8–11 $\left(\frac{24}{\text{I–V}}; \frac{7}{\text{II}}\right)$.

GASTROPODA 2, 4 $\left(\frac{10}{\text{I, III}}; \frac{6}{\text{II}}\right)$.

7. CENSUS OF MANURED PLOT.

The total number of invertebrates found in plot 2, in twenty-three samples, was 4485, or 15,100,955 per acre. Of these 2295 were insects, or 7,727,265 per acre.

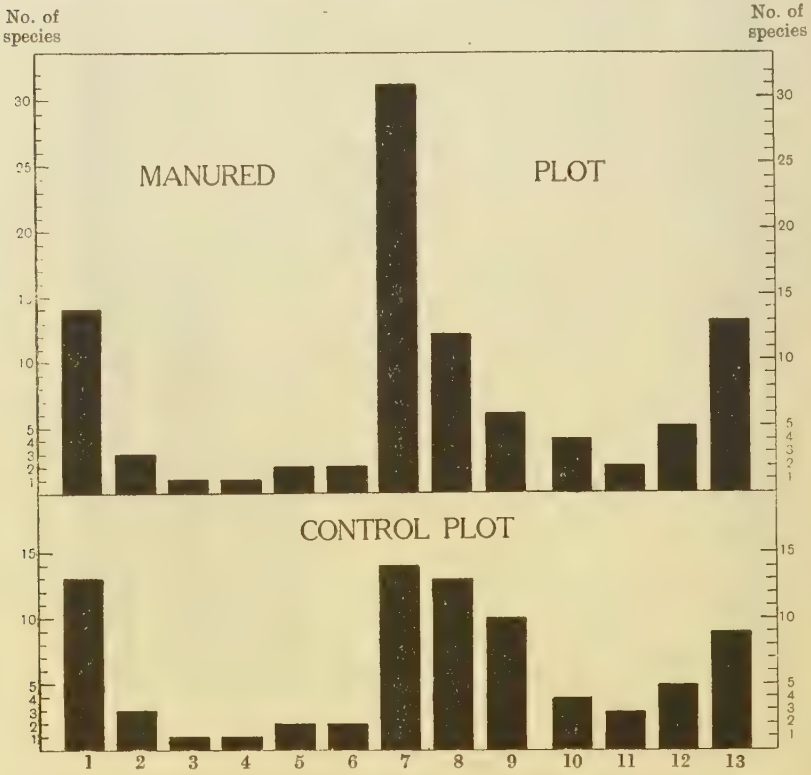
[1] The old term "Myriapoda" is used for convenience to include the classes *Diplopoda*, *Chilopoda* and *Symphyla*.



1, *Collembola*; 2, *Thysanura*; 3, *Orthoptera*; 4, *Thysanoptera*; 5, *Hemiptera*; 6, *Lepidoptera*; 7, *Coleoptera*; 8, *Diptera*; 9, *Hymenoptera*.

Fig. 5. Number of individuals in the different orders of insects in the manured and control plots.

The numbers per acre of the more abundant groups were as follows: *Oligochaeta* (*Limicolae*), etc., 3,609,424; *Formicidae* 2,946,125; *Collembola* 2,390,570; *Diplopoda* 1,367,002; *Oligochaeta* (*Terricolae*) 1,010,101; *Acarina* 531,986; *Chironomidae* (larvae) 515,151. The numbers of insects belonging to groups which are recognised as pests were: *Elateridae*



1, *Collembola*; 2, *Thysanura*; 3, *Orthoptera*; 4, *Thysanoptera*; 5, *Hemiptera*; 6, *Lepidoptera*; 7, *Coleoptera*; 8, *Diptera*; 9, *Hymenoptera*; 10, *Diplopoda*; 11, *Chilopoda*; 12, *Areinida*; 13, *Acarina*.

Fig. 6. Number of species in the different orders in the manured and control plots.

(larvae) 198,653; *Tipulidae* (larvae) 16,835; *Hepialidae* (larvae) 23,569. The numbers per acre in the different orders are shown in Fig. 5. The "probable error" in the total population per acre is ± 1,700,000, and in the number of *Elateridae* larvae per acre ± 22,000.

The number of species of insects which occurred in the samples was about 72 but the number may have been slightly higher, as all the larvae

found could not be exactly determined. The average number of insects per sample was 99·78.

The following orders occurred in the percentages given: *Collembola* 30·84; *Thysanura* 1·43; *Orthoptera* 0·17; *Thysanoptera* 0·26; *Hemiptera* 0·13; *Lepidoptera* 0·39; *Coleoptera* 10·25; *Diptera* 18·29; *Hymenoptera* 38·22.

The dominant order in respect of number of species present was the *Coleoptera*, with 31 species. The numbers of species in the different orders are shown in Fig. 6.

The most abundant species were *Myrmica laevinodis*, which made up 36·3 per cent. of the total insects; *Onychiurus ambulans*, 13·9 per cent. of the total; and *Onychiurus fimetarius*, 13·2 per cent. of the total.

Six species of *Myriapoda* (excluding *Symphyla*) occurred in this plot, and seventeen species of *Arachnida*.

8. Soil Fauna of the Control Plot.

The species of insects and other invertebrates present in the control plot are as follows:

INSECTA.

Collembola. Onychiuridae. *Onychiurus fimetarius* (Linn.) 1–10; *O. ambulans* (Linn.) 1–11; *Tullbergia quadrispina* (Börn.) 3, 4, 6, 10, 11.

Isotomidae. *Isotoma viridis* Bourl. 6, 9, 10; *I. minor* Schäff. 3, 4; *Folsomia quadrioculata* (Tullb.) 4; *Isotomurus palustris* (Müll.) 4, 10; *I. palustris* var. *aquatilis* 9.

Entomobryidae. *Entomobrya multifasciata* (Tullb.) 9; *Lepidocyrtus cyaneus* (Tullb.) 1, 10, 11; *L. albus* Pack. 3, 4, 9; *Orchesella villosa* (Geoff.) 1, 6, 9, 10, 12; *Heteromurus nitidus* Templ. 3, 4, 9, 10.

Collembola. All species 1–12 $\left(\frac{206}{\text{I–V}}; \frac{92}{\text{II}}\right)$.

Thysanura. Campodeidae. *Campodea staphylinus* Westw.; *C. gardneri* Bagn.; *C. fragilis* Meinert. Spp. 3–11 $\left(\frac{13}{\text{I–IV}}; \frac{6}{\text{II}}\right)$.

Orthoptera. Forficulidae. *Forficula auricularia* L. 2, 6 $\left(\frac{4}{\text{I–IV}}; \frac{2}{\text{II}}\right)$.

Thysanoptera. Spp. 6, 11 $\left(\frac{6}{\text{I}}\right)$.

Hemiptera. Jassidae. *Cicadula sexnotata* Fall. 9 $\left(\frac{1}{\text{I}}\right)$.

Cimicidae. *Lyctocoris campestris* Fall. 6 $\left(\frac{1}{\text{I}}\right)$.

Lepidoptera. Hepialidae. Unidentified larvae 2–4, 9 $\left(\frac{7}{\text{I–V}}; \frac{2}{\text{II, III, V}}\right)$.

Tineidae. Unidentified larva 8 $\left(\frac{1}{\text{II}}\right)$.

Unidentified larvae 3, 4, 9 $\left(\frac{6}{\text{I, II}}; \frac{5}{\text{I}}\right)$.

Coleoptera. Carabidae. *Clivina fossor* L. 4 $\left(\frac{1}{\text{II}}\right)$; *Bembidium guttula* F. 9 $\left(\frac{1}{\text{I}}\right)$.

Hydrophylidae. *Helophorus nubilus* F. 5 $\left(\frac{1}{\text{I}}\right)$.

Staphylinidae. *Homalota* spp. 2, 3, 6, 9, 10 $\left(\frac{6}{\text{I–IV}}; \frac{2}{\text{I, II}}\right)$; *Tachyporus hypnorum* F. 8

$\left(\frac{1}{II}\right)$; *Philonthus agilis* Grav. 9 $\left(\frac{1}{I}\right)$; *P. trossulus* Nord. 10 $\left(\frac{1}{I}\right)$; *Lathrobium longulum* Grav. 6, 9, 10 $\left(\frac{5}{II–V}; \frac{3}{III}\right)$; *Medon propinquus* Bris. 4 $\left(\frac{1}{I}\right)$; *Stenus subaeneus* Er. 5, 9 $\left(\frac{3}{I}\right)$; *Oxytellus insecatus* Grav. 2, 3 $\left(\frac{2}{I–III}\right)$.

CUCUJIDAE. *Silvanus surinamensis* L. 10 $\left(\frac{1}{I}\right)$.

ELATERIDAE. *Agriotes sputator* L. 3, 8 $\left(\frac{3}{I–III}\right)$.

CURCULIONIDAE. *Sitones humeralis* Steph. 2, 3, 8–10 $\left(\frac{10}{I}\right)$.

LARVAE AND PUPAE—CARABIDAE 4 $\left(\frac{1}{II}\right)$; STAPHYLINIDAE 2–4, 9–11 $\left(\frac{16}{I–IV}; \frac{8}{I}\right)$; SCARABAEIDAE 10 $\left(\frac{1}{I}\right)$; ELATERIDAE 1–12 $\left(\frac{49}{I–V}; \frac{16}{IV}\right)$; TELEPHORIDAE 10 $\left(\frac{1}{III}\right)$; CURCULIONIDAE, *Sitones humeralis* Steph. 9 $\left(\frac{1}{I}\right)$; Unidentified 4 $\left(\frac{1}{II}\right)$.

Diptera. CECIDOMYIDAE. *Campylomyza* sp. (larva) 2 $\left(\frac{1}{I}\right)$.

MYCETOPHILIDAE. *Sciara* sp. 9 $\left(\frac{1}{I}\right)$.

CHIRONOMIDAE. *Camptocladius aterrimus* Mg. Reared from larvae.

TIPULIDAE. *Trichocera fuscata* Mg. (larvae) 6–9 $\left(\frac{6}{I–III}; \frac{4}{I}\right)$.

BIBIONIDAE. *Dilophus febrilis* L. 9 $\left(\frac{1}{I}\right)$.

CHLOROPIDAE. Sp. 9 $\left(\frac{1}{I}\right)$.

Unidentified larvae of the following families also occurred:

CECIDOMYIDAE 1–6, 9–10 $\left(\frac{63}{I–V}; \frac{28}{I}\right)$; MYCETOPHILIDAE 2, 3, 9–11 $\left(\frac{23}{I–V}; \frac{10}{V}\right)$; CHIRONOMIDAE 3–5, 9, 10 $\left(\frac{8}{I–IV}; \frac{5}{II}\right)$; TIPULIDAE 1, 10, 12 $\left(\frac{5}{I, II}; \frac{4}{II}\right)$; SCATOPSIDAE 3 $\left(\frac{7}{II, IV}; \frac{6}{IV}\right)$; EMPIDAE 1–4, 9, 11 $\left(\frac{15}{I–IV}; \frac{8}{II}\right)$; SYRPHIDAE 3, 10 $\left(\frac{4}{I, II}; \frac{3}{I}\right)$; ANTHOMYIDAE 2, 10 $\left(\frac{7}{I}\right)$.

Hymenoptera. TENTHREDINIDAE. Unidentified larvae of two species 1, 6, 9 $\left(\frac{3}{I–V}; \frac{2}{I}\right)$.

CHALCIDIDAE. Three species, unidentified, 6, 10 $\left(\frac{3}{I–III}; \frac{2}{I}\right)$.

ICHNEUMONIDAE. *Pezomachus costatus* Bridge 9 $\left(\frac{1}{I}\right)$.

FORMICIDAE. *Myrmecina graminicola* Fabr. 10 $\left(\frac{1}{I}\right)$; *Myrmica laevinodis* Nyl. 3, 4, 6, 9 $\left(\frac{205}{I–V}; \frac{159}{III}\right)$; *Acanthomyops* (*Donisthorpea*) *nigra* L. 6 $\left(\frac{1}{I}\right)$.

ANDRENIDAE. *Andrena chrysosceles* Kirby 11 $\left(\frac{3}{III, IV}; \frac{2}{III}\right)$.

"MYRIAPODA."

DIPLOPODA. *Brachydesmus superus mosellanus* Verhoeff 1–5, 8–11 $\left(\frac{65}{I–IV}; \frac{26}{I}\right)$; *Cylindroiulus londinensis* var. *caeruleocinctus* (Wood) (= *C. londinensis* var. *teutonicus* (Pocock) of some records) 2–6, 9, 10 $\left(\frac{53}{I–V}; \frac{20}{I}\right)$; *Blaniulus guttulatus* (Bosc.) 1, 4–11 $\left(\frac{34}{I–V}; \frac{13}{V}\right)$; *Archiboreoiulus pallidus* Brade-Birks 1–12 $\left(\frac{25}{I–V}; \frac{7}{V}\right)$.

CHILOPODA. *Lithobius* sp. 6 $\left(\frac{1}{I}\right)$; *Geophilus longicornis* Leach 1–12 $\left(\frac{67}{I–V}; \frac{21}{IV}\right)$; *Geophilomorph* 2 $\left(\frac{1}{III}\right)$.

SYMPHYLA. Spp. 2–4, 9–11 $\left(\frac{19}{II–V}; \frac{6}{IV, V}\right)$.

ARACHNIDA.

Areinida. *Porrhomma pygmaeum* Pd. 3 $\left(\frac{1}{\text{III}}\right)$; *Centromerus bicolor* Bl. 3 $\left(\frac{1}{\text{II}}\right)$; *Trochosa terricola* Thor. 8, 9 $\left(\frac{2}{\text{I}}\right)$; *Stemonyphantes lineatus* L. 10 $\left(\frac{1}{\text{III}}\right)$; *Linyphia* spp. 9 $\left(\frac{1}{\text{I}}\right)$.

Acarina. ANYSTIDAE. *Anystis baccarum* L. 6 $\left(\frac{1}{\text{I}}\right)$.

GAMASIDAE. *Gamasus magnus* Kr. 1, 3 6, 9 12 $\left(\frac{14}{\text{I–III}}; \frac{9}{\text{I}}\right)$; *Gamasus* sp. (immature) 3, 4 $\left(\frac{2}{\text{I}}\right)$; *Pergamasus crassipes* L. 6, 10 $\left(\frac{7}{\text{I–III}}; \frac{6}{\text{I}}\right)$; *P. crassipes* var. *longicornis* 2, 10 $\left(\frac{4}{\text{I, II}}; \frac{3}{\text{I}}\right)$; *P. meridionalis* Berl. 11 $\left(\frac{1}{\text{II}}\right)$; *P. hamatus* Koch 6 $\left(\frac{1}{\text{III}}\right)$; *P. septrionalis* Oud. 6, 9, 12 $\left(\frac{6}{\text{I}}\right)$; *P. rumiger* Berl. 5, 10 $\left(\frac{2}{\text{I}}\right)$; *Pergamasus* spp. (immature) 3–11 $\left(\frac{13}{\text{I–IV}}; \frac{4}{\text{I, II}}\right)$.

OLIGOCHAETA (*Terricolae*) 1–12 $\left(\frac{136}{\text{I–V}}; \frac{56}{\text{II}}\right)$.

OLIGOCHAETA (*Limicolae*), **NEMATODA**, etc. 1–12 $\left(\frac{236}{\text{I–V}}; \frac{75}{\text{III}}\right)$.

ISOPODA 1, 3, 9–11 $\left(\frac{10}{\text{I–V}}; \frac{3}{\text{V}}\right)$.

GASTROPODA 1, 4, 10, 11 $\left(\frac{4}{\text{I–III}}; \frac{3}{\text{I}}\right)$.

Silvanus surinamensis L., which is recorded in the foregoing lists as having occurred once in the soil from each plot, is an introduced species which is usually recorded as having been found in stored foodstuffs, although Fowler (4) states that it has been taken under the bark of trees in Yorkshire, Epping Forest and Scotland. It seems doubtful if the specimens met with in the present instance could have been living in the soil; they may possibly have entered the soil in the laboratory before it was examined.

9. CENSUS OF CONTROL PLOT.

The total number of invertebrates found in plot 3, in twenty-three samples, was 1471 or 4,952,857 per acre. Of these 735, or 2,474,745 per acre, were insects.

The numbers per acre of the more abundant groups were as follows: *Oligochaeta* (*Limicolae*), etc., 794,612; *Collembola* 693,602; *Formicidae* 690,235; *Diplopoda* 595,959; *Oligochaeta* (*Terricolae*) 457,912; *Acarina* 215,488; *Chilopoda* 215,488. The numbers of insects belonging to groups recognised as pests were: *Elateridae* (larvae) 164,983; *Hepialidae* (larvae) 23,569; and *Tipulidae* (larvae) 16,835. The numbers per acre in the different orders are shown in Fig. 5.

The "probable error" in the total population per acre is ± 520,000, and in the number of *Elateridae* larvae per acre ± 44,000.

The number of species of insects which occurred in the samples was about 60 but, as in the other plot, this number might have been higher if all the larvae could have been exactly determined.

The average number of insects per sample was 31·95.

The following orders were represented in the percentages given: *Collembola* 28·14; *Thysanura* 1·78; *Orthoptera* 0·55; *Thysanoptera* 0·96; *Hemiptera* 0·27; *Lepidoptera* 1·91; *Coleoptera* 15·30; *Diptera* 22·13; *Hymenoptera* 28·96.

The dominant order in number of species present was the *Coleoptera*, with 14 species. The number of species in the different orders is shown in Fig. 6.

The most abundant species of insects were *Myrmica laevinodis*, which made up 27·9 per cent. of the total insects, *Onychiurus ambulans* 6·8 per cent., and *Onychiurus fimetarius* 6·5 per cent.

Seven species of *Myriapoda* (excluding *Symphyla*) occurred in this plot, and thirteen species of *Arachnida*.

10. Comparison of the Faunas of the Two Plots.

It is noticeable that in both the plots the *Oligochaeta* (*Limicolae*), *Formicidae* and *Collembola* were much the most abundantly represented groups, and that the *Diplopoda*, *Oligochaeta* (*Terricolae*) and *Acarina* were also very numerous in both plots. There was not very much difference between the numbers of *Elateridae* larvae in the two plots, the numbers being 198,653 per acre in plot 2, and 164,983 per acre in plot 3. It is also noticeable that the numbers of *Tipulidae* larvae and *Hepialidae* larvae are the same for both plots.

Other groups showed considerable difference in numbers between the two plots. *Diplopoda* occurred at the rate of 1,367,002 per acre in plot 2 and 595,959 per acre in plot 3, while *Trichocera* larvae occurred at the rate of 367,002 per acre in plot 2, but only at the rate of 23,567 per acre in plot 3, and *Chironomidae* larvae, which were found at the rate of 515,151 per acre in plot 2, were only found at the rate of 26,936 per acre in plot 3.

Most of the other groups occurred in somewhat greater numbers in plot 2: only one or two groups were found to be more plentiful in plot 3. Amongst the latter were the *Cecidomyidae* (larvae), 212,121 per acre in plot 3 and 195,286 per acre in plot 2, and the *Chilopoda*, 215,488 per acre in plot 3 and 208,754 per acre in plot 2, although the differences in these cases are not large enough to be of importance.

The equal or almost equal numbers of *Elateridae*, *Tipulidae* and *Hepialidae* larvae appears to show quite clearly that the continued use of farmyard manure does not cause an appreciable increase in the numbers of these injurious species although this manure appears to introduce or attract the injurious *Diplopoda* and certain non-injurious

species such as *Trichocera* and *Chironomidae* larvae, which probably are of some service in helping to open up the soil.

11. DISTRIBUTION IN DEPTH.

The depth at which the different organisms occurred was of considerable interest, and the samples were taken in five separate layers in order that their distribution might be accurately determined. This distribution was considerably affected by the ploughing of the plots, but seemed to be very little influenced by the operations of cultivation, harrowing and drilling.

In taking a sample of soil it was usually quite clear to what depth the ploughing had affected the soil, and as a rule a distinct change in the character of the soil was noticed in the fourth layer, taken between the five and seven-inch levels.

Of the total number of insects present, taking the whole period of the investigation, in plot 2, 78·7 per cent., and in plot 3, 50·3 per cent., occurred in the first two layers of soil, that is, between the surface and a depth of three inches. The percentages at the different depths were, for the manured plot: I 51·5; II 27·2; III 11·0; IV 6·4; V 3·8; and for the control plot: I 25·3; II 25·0; III 33·0; IV 11·1; V 5·5. Taking only the period from the commencement of the investigation in February until the plots were ploughed on October 13th, the percentages at the different depths were, for the manured plot: I 58·0; II 27·7; III 9·6; IV 2·5; V 2·2, and for the control plot: I 26·0; II 25·0; III 33·9; IV 10·2; V 4·8. Similarly, from the time of ploughing to the end of the investigation (October to January), the percentages at the different depths were, for the manured plot: I 8·7; II 24·3; III 20·0; IV 32·3; V 15·0, and for the control plot: I 16·6; II 24·0; III 22·2; IV 22·2; V 14·8.

It must be borne in mind, in comparing the percentages in the uppermost layer with those in the other layers, that the volume of soil in this top layer was considerably less than in the other layers, as it consisted of the soil between the surface and a depth of one inch below the surface only, while the remaining layers consisted of the soil for a depth of two inches.

Most groups of insects, etc., considering the period of the investigation as a whole, occurred in the largest numbers in the second layer, with a rather lower percentage in the first. The third usually contained a distinctly smaller percentage than the second, quite commonly being from one-half to one-third the number, while the fourth layer usually stood in about the same relation to the third, the difference being in

some cases even greater. The same relation existed again between the fifth and fourth layers. The fact that the figures given above do not coincide with this is due chiefly to the distribution of the ants, which occurred on two occasions in large numbers, owing to the sample containing part of a nest. One of these nests occurred in the first layer of a sample from the manured plot, and the other in the third layer of a sample from the control plot, before the plots were ploughed.

In Fig. 7 the *Formicidae* have been omitted. This diagram indicates very clearly that the *Insecta*, "*Myriapoda*" and *Oligochaeta* (*Terricolae*) probably penetrate to a greater depth than nine inches.

A few groups showed noticeable variations from the above general rule. The *Acarina*, *Cecidomyidae* (larvae), *Chironomidae* (larvae) and *Trichocera* (larvae) were found to occur in much larger proportions in the upper layer than in the second, and very few occurred below the five-inch level. With the *Symphyla* the usual proportions per layer were practically reversed, much the greatest proportion of this group occurring in the fourth and fifth layers.

After the plots had been ploughed the effect of the ploughing on some of the groups of invertebrates was very clear for some time. Taking the numbers of *Collembola* for example, from the beginning of the investigation in February to the time of ploughing in October, the percentages in the five layers were: I 29·0; II 44·0; III 19·8; IV 3·0; V 4·1 in the manured plot, and I 28·6; II 46·8; III 16·1; IV 4·6; V 3·6 in the control plot.

For the period from the time of ploughing to the end of the investigation, the percentages were: I 2·1; II 12·2; III 20·1; IV 44·6; V 20·9 in the manured plot, and I 14·3; II 14·3; III 28·6; IV 28·6; V 14·3 in the control plot.

In the case of the *Elateridae* larvae, taking the whole period of the investigation, the percentages at the different depths were, in the manured plot: I 1·7; II 18·6; III 20·3; IV 32·2; V 27·1; and in the control plot: I 12·2; II 18·4; III 26·5; IV 32·6; V 10·2. Taking only the period from the commencement of the investigation to the time of ploughing the percentages at the different depths were, for the manured plot: I 2·8; II 30·5; III 27·8; IV 22·2; V 16·7; and for the control plot: I 11·6; II 20·9; III 27·9; IV 34·9; V 4·7. After the plots had been ploughed, taking the period from the time of ploughing to the end of the investigation, the percentages at the different depths were, for the manured plot: I nil; II nil; III 8·7; IV 47·8; V 43·5; and for the control plot: I 16·7; II nil; III 16·7; IV 16·7; V 50·0.

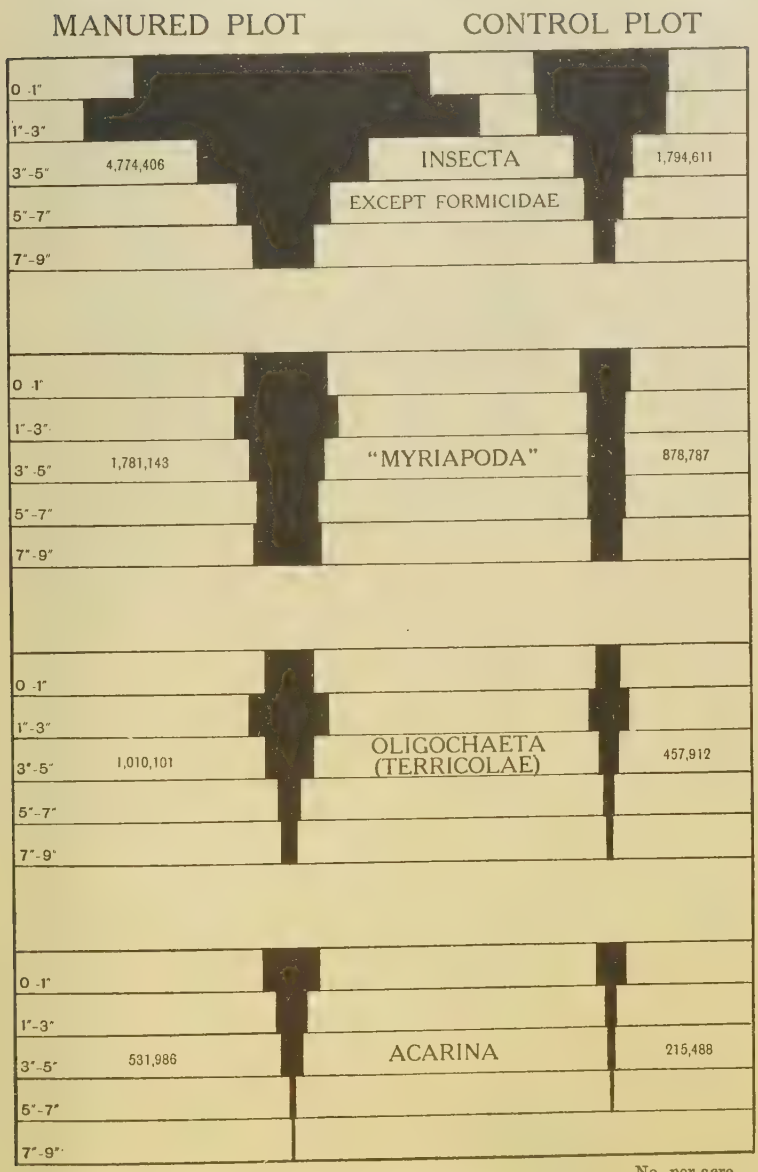


Fig. 7. Distribution in depth of the more important groups in the manured and control plots.

Effects of a similar nature due to the ploughing were observed in some other groups, while with others, such as the *Acarina*, the effect was very little marked, as they appeared to regain the upper layers after being buried by the plough.

Although the percentage at the different depths varied somewhat between the two plots, the general distribution of the insects, etc., was very little different in one plot from that in the other.

No seasonal variation in the distribution in depth of the soil fauna was observed.

12. Comparison with Soil Fauna of Pasture Land.

It is not possible to compare very fully the soil fauna found in the present investigation with that previously found in the examination of permanent pasture (10) owing to the considerable difference in the conditions under which it was existing. The localities in which the work was carried out are widely separated, being in Hertfordshire and Cheshire respectively, and the soil and weather conditions differ considerably.

In pasture land few insects were found at a greater depth in the soil than two inches, and none at a greater depth than six inches. The depth to which insects penetrated into the soil was considered to be chiefly influenced by four factors—depth to which their particular food occurs; aeration; moisture; and temperature of the soil. It was shown that in permanent pasture these four factors all tended to restrict the insects to the superficial layers of soil.

In the present instance these four factors influence the fauna differently, owing to the field being under cultivation. The periodical turning over and stirring of the soil makes it fairly certain that the soil, to the depth to which the implements of cultivation penetrate, will be fairly uniform in composition, and the aeration and drainage of the soil will be more favourable owing to its greater looseness.

In arable soil the conditions are thus much more favourable to deeper penetration by the insects. The number of insects in the control plot is less than was found in the pasture (3,586,088 per acre), but the number in the manured plot is considerably greater.

13. Relation of Soil Fauna to Soil Nitrogen.

In order to determine the importance of the soil fauna as a reserve and source of nitrogen, the nitrogen content of several groups of insects, etc., was estimated, and from these figures it is possible to obtain an estimate of the amount of nitrogen in the whole fauna.

The nitrogen content of the following groups was obtained: *Elateridae* larvae, *Collembola*, *Formicidae*, *Oligochaeta* (*Terricolae*), *Myriapoda* and *Oligochaeta* (*Limicolae*), the percentage of nitrogen in the dry weight being: *Elateridae* larvae 10·65 per cent.; *Collembola* 11·18 per cent.; *Formicidae* 10·92 per cent.; *Oligochaeta* (*Terricolae*) 9·4 per cent.; *Myriapoda* 4·88 per cent.; *Oligochaeta* (*Limicolae*) 6·26 per cent.

The total weight of nitrogen per acre contained in the bodies of the above groups in the manured plot is approximately: *Elateridae* larvae 206·0 gm.; *Collembola* 8·5 gm.; *Formicidae* 306·6 gm.; *Oligochaeta* (*Terricolae*) 4626·0 gm.; *Oligochaeta* (*Limicolae*) 97·0 gm.; *Myriapoda* 1864·9 gm.

Assuming that the remaining insects are of the same average nitrogen content, the total nitrogen of all the insects in an acre of the manured plot is 687·7 gm.

The nitrogen contained in the *Oligochaeta* (*Terricolae* and *Limicolae*) and *Myriapoda* is 6587·9 gm. per acre of the manured plot. These groups include 6,400,668 of the 7,373,730 invertebrates other than insects, the remaining 973,062 consisting chiefly of *Arachnida*, with some *Isopoda* and a few *Gastropoda*. Assuming their nitrogen content to be the same as that of the same number of insects, it would be 74·0 gm. giving a total of 6661·9 gm.

The total nitrogen of the fauna of an acre of the manured plot is thus 7349·6 gm. or 16·2 lbs.

In the control plot the nitrogen contained in the bodies of the same groups is: *Elateridae* larvae 169·0 gm.; *Collembola* 2·4 gm.; *Formicidae* 71·5 gm.; *Oligochaeta* (*Terricolae*) 2128·0 gm.; *Oligochaeta* (*Limicolae*) 21·4 gm.; *Myriapoda* 920·1 gm.

Again assuming that the remaining insects are of the same average nitrogen content, the total nitrogen of all the insects in an acre of the control plot is 313·3 gm.

The nitrogen contained in the *Oligochaeta* (*Terricolae* and *Limicolae*) and *Myriapoda* is 3069·5 gm. per acre of the control plot.

These groups include 2,131,311 of the 2,478,112 invertebrates other than insects in an acre of the manured plot. Assuming that the remaining 346,801 invertebrates have the same nitrogen content as the same number of insects, their nitrogen content is 26·4 gm.

The total nitrogen contained in the bodies of the fauna of an acre of the control plot is thus 3409·2 gm. or 7·5 lbs.

These amounts of nitrogen are equivalent to the nitrogen contained in 103·6 lbs. and 48·0 lbs. of nitrate of soda in the manured and control plots respectively.

It appeared possible that the introduction of insects, etc., in an application of farmyard manure, and their subsequent death and decay with gradual liberation of nitrogen, might account for the effects of an application of farmyard manure being noticeable for a considerable time afterwards. The quantity of nitrogen contained in the fauna seems, however, to be too small to be of great importance in this way, even although the manured plot in this case had received farmyard manure annually for 77 years.

Although the bodies of the invertebrate fauna of the soil contain quite an appreciable amount of nitrogen, there can scarcely be any loss or gain of nitrogen due to them. The *Oligochaeta*, *Myriapoda* and other groups which live and die in the soil, eventually return to it, at their death, all they have taken from it. Although winged insects may leave a plot in which their larvae have fed, this is probably balanced by other insects migrating to the plot and dying there, whose larvae have fed elsewhere.

14. The Function of the Invertebrate Fauna in the Soil.

Since the work of Darwin (1) and others (6), (14) the importance of the earthworms in the soil has been widely recognised, the uniformity and loose texture of the surface soil being attributed largely to them. By means of their burrows air and water are enabled to penetrate the soil, and their habit of drawing leaves, blades of grass and other vegetable remains into their burrows adds to their importance.

A considerable proportion of the damage done to land by floods is considered to be due to the flooding out of the earthworms, so that the surface soil remains compacted and vegetation languishes until a new immigration of earthworms has restocked the soil.

Some authors (3), (6), (7), (13) consider that, in addition to the mechanical work of loosening the soil and assisting aeration and drainage, the earthworms, by the passage of considerable quantities of soil through their bodies, render the mineral substances more readily available for plants. On the other hand, the results of other experiments have tended to disprove this theory (12).

It has also been stated that by following the burrows of earthworms, the roots of plants are able to penetrate to a greater depth than would otherwise be the case, although this is denied by other workers (2).

The work of insects, insect larvae and other invertebrates in the soil is probably similar to that of the earthworms (7) in assisting in the

aeration and drainage of the soil. Since they pass smaller quantities of soil through their bodies than in the case of earthworms, they probably do not affect the soil to the same extent.

Kostitcheff, in his work on the Russian "Black earth" (8), states that the action of worms and insects in the soil is of great importance in assisting in the breaking down of vegetable matter and the formation of humus. In damp places where worms and insects are unable to live, the vegetable matter is broken down very much more slowly, and peat, in which the vegetable matter still retains a certain amount of structure, is formed instead of an amorphous humus. He does not agree with Darwin with regard to the importance of earthworms in bringing soil from the lower levels to the surface.

In experiments carried out on earthworms, millepedes and *Sciara* larvae Kostitcheff (9) found that they had little effect in accelerating the decomposition of dead leaves, but he considers that after being once passed through the animal, the material is then acted on by fungi and bacteria, and again made available as food for the worms and insects, and in this way the vegetable matter is eventually completely broken down.

Darwin estimated that earthworms brought to the surface of the soil, in their "casts," sufficient earth to form annually a layer 0·2 inch in depth, or dry earth weighing ten tons per acre, and that in 50 years the upper ten inches of soil is completely turned over by them.

Hensen, quoted by Darwin, calculated that there were 53,767 earthworms in an acre of garden soil, and found open burrows to the number of 196,020 per acre, although Darwin states that he has seen them much more numerous. Hensen estimated that there would be half as many earthworms in an acre of cornfield as in garden soil. Darwin, who obtained the number and weight of the "worm-casts" over certain areas, did not give any relation between the number of "casts" and the number of worms present.

In the present investigation the numbers of worms found, 1,010,101 and 457,912 in the manured and control plots respectively, are very much above Hensen's estimates.

SUMMARY.

1. Samples of soil were taken from two of the plots at the Rothamsted Experimental Farm and all insects and other invertebrates were recorded together with the approximate depths at which they occurred.

2. One of these plots (plot 2) has received 14 tons of farmyard manure per acre per annum since 1843; the other (plot 3) has received no manure of any kind since 1839. This difference in treatment had a very marked effect on the number of insects present.

3. Twenty-three samples of soil were examined from each plot, each sample being a cube $9 \times 9 \times 9$ inches. The soil in each sample was removed in five layers, so that it was possible to determine the approximate depth at which the specimens occurred.

4. There were, in round numbers, 15,100,000 invertebrates per acre, of which 7,720,000 per acre were insects, in plot 2, and 4,950,000 invertebrates per acre, of which 2,470,000 per acre were insects, in plot 3.

5. The greatest number, both of insects and of other invertebrates, occurred in the upper three inches of the soil, but some species were found in larger numbers at a greater depth, the greatest number of *Elateridae* larvae being found at a depth of five to seven inches, and of *Symphyla* at a depth of seven to nine inches.

6. Some species, such as the larvae of *Chironomidae* and *Trichocera*, were practically confined to the plot which had received farmyard manure, plot 2, while other species, such as the *Collembola*, *Onychiurus ambulans* and *O. fimetarius*, although they occurred in both plots, were considerably more numerous in plot 2.

7. Injurious insects, such as the larvae of *Elateridae*, *Tipulidae* and *Hepialidae*, appeared to be little affected by the different manurial treatment of the two plots, and occurred in practically equal numbers in the two plots.

8. Although 198,653 and 164,983 *Elateridae* larvae per acre occurred in plots 2 and 3 respectively, they did not produce any appreciable effect on the crop.

9. An attempt was made to estimate the amount of nitrogen contained in the bodies of the soil fauna, and it was found to be 7349·6 gm. or 16·2 lbs. and 3409·2 gm. or 7·5 lbs. in plots 2 and 3 respectively. It is unlikely that there is any appreciable loss of nitrogen from the soil due to the migration of winged members of the fauna.

10. The worms, insects and insect larvae are beneficial in loosening the soil and facilitating aeration and drainage.

11. The net results of these observations show that, although the introduction of farmyard manure greatly increases the invertebrate population of the soil, the latter organisms are saprophagous and are not directly injurious to the growing crop. Such injurious organisms as are present occur in approximately equal numbers whether the land be

manured or not. The most notable exception to this generalisation is met with in the *Diplopoda*, whose numbers are increased by about 200 per cent. in the manured plot.

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ON THE LIFE HISTORY OF "WIREWORMS" OF THE GENUS *AGRIOTES*, ESCH., WITH SOME NOTES ON THAT OF *ATHOUS HAEMORRHOIDALIS*, F.[1]

PART III

By A. W. RYMER ROBERTS, M.A.

(*Zoological Laboratory, Cambridge.*)

(With 1 Text-figure and Plates XIII, XIV.)

AGRIOTES SPUTATOR, L.

Of the life history of this species as distinct from that of other members of the genus, not much is as yet known. Köllar in 1837 referred to the larva as feeding on lettuces and describes it as being "light yellow, from six to seven lines long, of the thickness of a pigeon's quill." Curtis(5) (p. 167) and other writers of the nineteenth century seem to have taken their accounts of the species from Köllar, whose details are so very meagre. Adrianov(1), however, as has already been mentioned, obtained the ova and young larvae in Russia in 1914. But he does not appear to have grown the larvae for more than a year and his description of the experiments he made provides no means of distinguishing this larva from others of the same genus.

My own attempts to breed this species from the egg have, from one cause or another, not been very fortunate, though I have obtained ova from my breeding pots in three separate years. The longest-lived brood did not quite survive two years (1916–1918), but from it a few points have at least emerged; firstly, that the rate of growth of the larva within the time named was almost the same as that of *A. obscurus* of the same age, and secondly particular features of the structure have been observed, providing the link connecting the young larvae with older larvae which were taken in the field and from which beetles were bred.

In regard to the first point, only two larvae were obtained after the second winter, but if these can be considered to be of normal size, as is probable, and if their future rate of growth would have corresponded with the past rate, then it appears that there is but little difference in

[1] A grant has been received for publication of this paper.

length between *A. obscurus* and *A. sputator* at the same age. Now the larva of *A. sputator* is full-fed when of a length of 16–17 mm. and assuming that its rate of growth is the same as that of *obscurus* it should attain this size by the end of its third year of life. Allowing then for the period at the end of the larval stage, during which but little increase in length takes place, it may be concluded that pupation occurs during the fourth year and that the mature beetle emerges four years after the hatching of the egg, or one year less than the time taken in the life cycle by *A. obscurus*. We have as yet little accurate information as to the duration of the life cycle in other species of the genus, but Xambeu states(12) that *A. sordidus* occupies only one year in the larval stage, while Hyslop found(6) that *A. mancus*, an American species, in the northern United States of America, pupated after three years. It seems probable, therefore, that the duration of the life cycle varies much between different species of the genus and the evidence so far is in favour of *A. sputator* accomplishing it in one year less than *A. obscurus*.

Other points in the life history coincide closely with those of *A. obscurus*, though there may be differences not yet observed. The larvae of both moult twice in the year, they pupate at the same time and the beetle emerges from the pupal condition also at the same time. Köllar says that the duration of the pupal stage is only fourteen days. This has not been verified and may perhaps not apply to the climatic conditions of this country.

No difference in regard to choice of soil has been observed, though *A. sputator* appears to require milder conditions, being comparatively scarce north of Cheshire and Norfolk and rare in Scotland, while it is not known in Ireland. On the continent of Europe also, though its range generally coincides with that of *A. obscurus*, it becomes rather scarce in the centre of Sweden (Thomson) and in Finland (du Buysson), while *A. obscurus* occurs as far north as Lapland.

In regard to the morphology of the larva certain external features, specified in detail later on, have emerged from comparison between the two species, differentiating them from one another. In the first instar, at least, the characters of this species separate it also from *A. acuminatus*, Steph. (*sobrinus*, Kies.). It has not yet been possible to compare it with *A. lineatus*, L. and *A. pallidulus*, Ill. The first-named must, however, from Beling's description, closely resemble *A. obscurus*, while *A. pallidulus* according to the same author seems to lack the sensory pits on the 9th abdominal segment and to resemble rather the larva of *Dolopius marginatus*.

Ovum.

Generally broadly ovoid but varies considerably in both shape and size. Average dimensions of ten ova ·54 mm. × ·43 mm. and therefore slightly smaller than those of *A. obscurus*. One ovum found was almost bean-shaped and measured ·475 × ·43 mm. In this species also the shell is transparent and almost smooth, the whole appearing to be milky white from the colour of the contained yolk and embryo.

First Larval Instar.

In general appearance the larva is extremely like *A. obscurus* at the same age. The average length during the first day after hatching is just under 2 mm. (1·9), ranging from 1·25 to 2·25 mm. in a dozen specimens; the breadth across the prothorax about ·25 mm. The ventral surface is flat, the dorsal arched, but less so than in older larvae. Colour milky white. It is difficult to see any material difference in the sculpture of the dorsal surface in preserved specimens, but in life the young *sputator* is a trifle more rugose and punctulate.

The head is about equally long and broad, measuring the length from the base of the mandibles to the occiput and the breadth across the broadest part, a little anterior to the middle. It is longer than either the meso- or meta-thorax. As in *A. obscurus*, the mandibles (Text-fig. 1 *a*) are brown at the apex and broader in proportion to their length in the first than in the final instar. The nasale or clypeal process is represented by an entire rounded projection above the mouth. Beneath this, traces of the sub-nasal process are visible, usually as a minute notched process at the base of the nasale, with one or two smaller rounded thickenings of the chitin beyond the lateral margins of the nasale. In the antenna, the third or supplementary segment is longer than the conical ventral process at the apex of the second segment, but much less so than in mature larvae. At this stage, it is also longer in proportion to the whole antenna than in older larvae.

Of the setae with which the tergites are furnished, the posterior row is, as usual, longer than the anterior, but they are not so long as the segments to which they belong. Those of the two rows on the prothorax, anterior and posterior, are of about equal length. The setae of the head and also those surrounding the cauda are shorter than these. Subsequent measurements of the setae of *A. obscurus* at the same stage show that the relative proportions previously given (Pt. II, p. 195) must be amended, those on the abdominal segments being the longest, while

those surrounding the cauda and those of the head are considerably shorter, as in *A. sputator*.

The shape of the spiracles at this stage is very variable and does not give any certain means of separation of the species, though the number of teeth on either side of the orifices has been found to be less in *sputator* than in *obscurus*. In the former these number five in the thoracic, four or five, generally four, in the abdominal spiracles. As in *obscurus* the orifices of the spiracle frequently appear to be separately margined by a raised border, most evident at the sides, very fine behind and lacking in front. In reality, however, as may be seen under a high-power objective, the margin is patterned on the surface much as in older larvae.

Text-fig. 1. (*a*) *Agriotes sputator*, L. Mandible of larva in first instar. Magn. cir. × 700.
(*b*) *Athous haemorrhoidalis*, F. Mandible of larva in late instar, seen from above.
(*c*) *Athous haemorrhoidalis*, F. Clypeal process or nasale of larva. Magn. cir. × 130.
(*d*) *Agriotes acuminatus*, Steph. Mandible of larva in first instar. Magn. cir. × 770.

The median area of the septum between the two orifices is lighter in colour and less strongly chitinized but it is in like manner furnished with a corrugated pattern, corresponding to the teeth at the sides of the orifice. The ventral orifice is most frequently smaller than the dorsal one, though considerable variation in size and shape occurs.

Towards the apex of the 9th abdominal segment the same constriction is apparent in both this species and *A. obscurus* at hatching, but disappears later during the first instar. It may be no more than a coincidence, but among the specimens examined the margins of the sensory pits on the 9th abdominal segment are usually coloured brown at or shortly after hatching, whereas in *obscurus* the margins are only to be delimited with difficulty until a much later age.

The cauda, though colourless at first, later becomes slightly tinted with yellow. Its shape affords a slight means of differentiation, for while that of *obscurus* is blunt, it is distinctly pointed, though quite short, in *sputator*.

THIRD INSTAR.

In the early part of the third instar the larva is about 6·5 mm. in length (nearly the same as *A. obscurus* at the same age), and of a pale yellow colour, though this appears to vary somewhat with the individual. In section it is considerably more rounded than specimens in the first instar, but is slightly flatter on the dorsal and ventral aspects than at the sides. In general the larva may be distinguished from that of *A. obscurus* by its coarser punctuation and by its longer and proportionally narrower spiracles.

The head is rather smooth and its setae of the posterior are longer than those of the anterior row. Length of segments of the antennae as 25:13:21, taking the basal, second and supplementary segments. Eyes situated in a line with the anterior pair of setae and behind the antennae. The mandibles appear to be somewhat sharper-pointed and more curved on the outer margin than in *A. obscurus* at the same age. The nasale is distinctly tridentate, with the middle tooth extending considerably further forward than the two lateral teeth. The sub-nasal process is not well defined: in one specimen examined it consists of five rounded teeth borne in an almost straight line at the base of the nasale, the middle tooth being a little more prominent than the rest.

The tergites are coarsely punctured with irregularly-shaped punctures, while the anterior margin of each of the abdominal tergites 1–8 bears a fine and close granulation, which extends backwards as far as the spiracles. This granulation is also present on the 9th abdominal tergite, though the remainder of the tergite is less strongly punctured than that of any other abdominal segment. It is absent from the pronotum, but present on the meso- and meta-notum, where is extends to the anterior row of setae.

The sternites are more sparsely punctured than the tergites but bear a few punctures and also a few somewhat irregular transverse rugae. The posterior margin of the prothoracic sternite behind the coxae and the whole of the meso- and meta-thoracic sternites bear fine granulations.

All the setae are yellowish. Those of the pronotum are about equal in length as between the anterior and posterior rows, while in the first eight abdominal segments the posterior row is the longer. None is as long as the segment to which it belongs. The ventral setae are short.

The spiracles are now distinctly elongate, the ventral orifice being often a little shorter than the dorsal. They are longer and proportionally narrower than those of *A. obscurus* at the same age. At either side of each orifice the teeth or corrugations number 14 to 16 in the thoracic, 10 or 11 in the abdominal spiracles. The marginal rims are brown.

The 9th abdominal segment appears to be narrowed to its apex somewhat more gradually than that of *A. obscurus*, being widest in the region of the sensory pits. These latter are small, round and surrounded with a brown margin. The cauda is short, but a trifle more acute than that of *A. obscurus*. At this stage it is slightly yellower than the surrounding area of the cuticle.

Final Instar.

In general the larva closely resembles that of *A. obscurus*, already described in Part II(9). It does not, however, attain the same size, being full fed when of a length of 16–17 mm. and a breadth of 1–1·5 mm. Many specimens are a little darker in colour but this character is not reliable.

Head with a few shallow punctures and short irregular longitudinal striae above and beneath. *Antennae* differ from those of the early stages in having the third or supplementary segment shorter in proportion to the other segments and especially the basal one, the proportion being as 25:9·5:12·75 from basal to third segment. *Mandible* somewhat narrower in proportion to its length than that of *A. obscurus*, but the difference is not very marked. Both species have the posterior one-third on the ventral surface minutely and rather closely punctulate.

The anterior portion of the *cephalic plate*, which overlies the base of the mandible on either side, is in *A. sputator* rather more pointed at the apex than in *A. obscurus*.

By an unfortunate mistake the semi-membranous lining of the *palate* was described in Part II (p. 206) as the floor of the mouth. The "anterior margin" appears to represent a suture found a short distance behind the sub-nasal process, while the tufts of bristles referred to are in reality a portion of those at the anterior margin of the cephalic plate, on its ventral side. The real floor of the mouth is a membranous structure lined with fine hairs and situated behind the base of the laciniae, above (dorsal to) the mentum.

The anterior portion of each *tergite*, with the exception of that of the prothorax, is minutely granulate (Plate XIII, fig. 1*x*). On the meso- and meta-nota and abdominal tergites 1–8 the granulations extend in a transverse band from the intersegmental membrane to a line of minute pores

just anterior to the anterior row of setae and to the spiracles in the case of the abdominal segments. On the 9th tergite they extend almost to the anterior margin of the sensory pits.

The main portion of each tergite is, compared with that of *A. obscurus*, distinctly rugose and coarsely pitted with rather deep and irregularly shaped punctures (Plate XIII, fig. 1*c*). The posterior portion of the meso- and meta-thoracic and of the 1st to the 8th abdominal tergites, as well as both anterior and posterior margins of the pronotum, are occupied by similar borders of longitudinal striations as in *obscurus*. The prothorax and 9th abdominal segment are dorsally somewhat smoother than the other segments of the body but have more and deeper punctures than the corresponding segments of *A. obscurus* and also some irregular rugae.

Of the setae with which the tergites are furnished; the longer ones of the two rows on the prothorax are about equally long, but in the case of each of the succeeding segments, up to the 8th abdominal segment, those of the posterior are longer than those of the anterior row. The same proportion in the respective length of the setae applies to *A. obscurus*, though it is not made clear in my description (Pt. II, pp. 200–201). The chaetotaxy of the 9th abdominal segment is also generally the same, though an extra seta near the posterior margin of each sensory pit occasionally present in *A. sputator* and shown in Plate XIII, fig. 1*a*, has not been observed in *A. obscurus*.

In the *pleurite* of the meso-thorax the granulations extend from the anterior margin, along the ventral margin of the spiracle to its posterior end, the dorsal side being smooth. In the epipleurites of the abdominal segments there is also a little granulation at the anterior end of each, the remainder of the surface being punctured similarly to the sternites. On the ventral surface the *prosternum* is almost smooth, but its posterior portion as far as the coxae is granulate and the granulations extend on the posterior side of the coxae themselves for half their length. The whole of the meso- and meta-sterna are granulate and the basal half of the coxae belonging to the same segments have granular areas corresponding to those of the anterior coxae. Similar granulation of areas on the ventral surface of the thorax has been found in *A. obscurus*.

The *abdominal sternites* are more sparingly punctured than the tergites and bear, in addition to the punctures, a number of irregular, more or less transverse, furrows. On the anterior margin of each sternite there is a band of granulations, as on the tergites, extending backward as far as the anterior row of setae. On the 9th abdominal sternite the granulation extends almost to the base of the pseudopod, while that portion

of the sternite which lies posterior to the setae bears a few shallow punctures and rugae only.

The *spiracles* (Plate XIII, fig. 1*d*) differ from those of *A. obscurus* in being actually longer (in spite of the smaller size of the larva) and also in being longer in proportion to their breadth. Their sides also are more nearly parallel, the spiracles of *A. obscurus* being widened more considerably anteriorly. Length of the first abdominal spiracle about ·137 mm. and maximum breadth about ·056 mm., while in the larger larva of *A. obscurus* the corresponding measurements are ·125 mm. and ·085 mm. As might be expected the number of teeth or corrugations on either side of the spiracular orifices is also somewhat greater, numbering about 51 in the thoracic and 45 in the abdominal spiracles. Malformation of single spiracles occurs occasionally. Mr Terzi's figure (Plate XIII, fig. 1*e*) clearly shows the nature of the malformation in one specimen while another similar one has also been met with.

Cauda short and generally not very acute: on the average it appears to be slightly sharper than that of *obscurus*.

Apart from the points set out above the description of *A. obscurus* in the late larval stages would serve equally well for this species. As will have been observed, the most salient differences between the two species, apart from size, rest in the sculpture of the cuticle and in the shape of the spiracles. The following comparative table shows the nature of the principal distinctions:

A. obscurus.	*A. sputator.*
Tergites nearly smooth, glossy: bearing shallow furrows, chiefly longitudinal, of variable length: punctures sparse and shallow.	Tergites rather rugose, dull: rugosities irregular, frequently transverse: punctures more numerous, wider and deeper.
Area anterior to spiracles, both dorsally and ventrally, almost smooth.	Area anterior to spiracles, dorsally and ventrally, finely granulate.
Tergite of 9th abdominal segment almost smooth, but bearing a number of shallow furrows irregularly disposed: a few shallow punctures towards the apex.	Tergite of 9th abdominal segment slightly rugose and punctulate: finely granulate anterior to sensory pits.
Spiracles shorter, widest at anterior end.	Spiracles long and narrow; scarcely wider at anterior end.

Pupa.

In general the pupa resembles that of *A. obscurus*, but is smaller, has the prothorax more elongate and differs in several other characters to be specified below.

In length it is about 8 mm. with a breadth of about 2·5 mm. across the thorax. The anterior thoracic spines are attached just above the

imaginal eye, which early shows through the integument; they are long and tapering and terminate in a fine brown bristle. The suture between head and prothorax extends from the base of each antenna over the eyes of the adult and is continued ventrally in a semicircle above the vertex of the head.

The prothorax is longer than broad, rounded at the sides, swollen, slightly striated transversely. As in the adult, it is deeper anteriorly and has on the dorsal surface a slight median groove which is deepened posteriorly. The antennae reach to the intermediate pair of legs at the point where the femora are flexed against the tibiae. Their segments are longer than broad, enlarged towards the apex of each segment, except the last, and bear blunt tubercles on either side. The posterior angles of the prothorax are produced into long outstanding spines, fleshy at the base and continued in fine curved brown setae arising from the outer side of the fleshy base. The median spines, situated in many Elaterid pupae one on either side of the medio-dorsal groove, are entirely absent, even the tubercles, visible in *A. obscurus*, being lacking.

Elytral sheaths just reaching to the 5th abdominal sternite, bearing at their apices a small upturned blunt hook. The first abdominal spiracle is concealed beneath the base of the wing-sheath.

Both tergites and sternites of each abdominal segment from the 2nd to the 6th are somewhat sinuate laterally and are produced at their posterior margins into a kind of flange. In life this prolongation of tergites and sternites serves to conceal the spiracles, which are situated in the pleura of each segment. The tergites and sternites of the 7th and 8th segments are scarcely produced laterally and leave the spiracles exposed.

Ventrally, the 7th abdominal sternite is longer than the others and almost paraboloid in shape, though it has in the male pupa, at least, a slight angle at the point where it overlaps the 8th sternite.

The sexual differences are manifest on the ventral surface of the 9th segment as in *A. obscurus*.

The terminal processes arise laterally and project from the body at an angle of some 45°. The basal portion of each is cream-coloured and fleshy, the apical brown and produced into a spine. The spines are neither so long nor so sharply pointed as are those of the prothorax.

AGRIOTES SOBRINUS, Kies. = *ACUMINATUS*, Steph.

Of this species very little is known. Ova were obtained in 1918 from the soil of a pot within which the beetles had been confined and a few larvae were obtained from these ova. But the eggs laid in the pot appear

to have been few and no larvae were reared beyond the first instar. There appear, however, to be no other records of any part of the life history of this insect apart from the adult stage, so that it seems desirable to add what little information is available while dealing with other members of the genus.

In Britain this species is a southern and midland one even more than the last and though generally fairly common in places where it occurs, it is local in its distribution. Abroad it is known over the greater part of the continent of Europe, extending to the Western Caucasus (du Buysson).

The adult is generally referred to as frequenting woody places and flowers, especially those of *Umbelliferae*. Though the beetles are commonly taken in fields and on roadsides away from woods, it is possible that their proper breeding habitat may be in woods, a possibility which has some slight confirmation in the fact that none have yet been bred by the writer from larvae taken in agricultural land.

Ovum.

Outline figures of the ova have already been given (Pt. I, Text-figs. 1 and 2). According to the small amount of material examined, their shape is very variable, but they appear to show a somewhat greater tendency to be pointed at one end than do the ova of *A. obscurus* or *A. sputator*. Average dimensions of six ova ·56 × ·44 mm., the largest measuring ·61 × ·47 mm. In general ovoid, white and somewhat shining, though under the microscope the shell may be seen to be minutely pitted.

Ova were found on the 9th, 10th and 13th July at a depth of ¾ to 1½ inch below the surface of the soil. The first larva hatched on the 10th August, so that the incubation period is probably at least a month.

First Larval Instar.

On hatching the larva is from 2·5–3·0 mm. in length, semi-transparent, milky white to slightly buffish in colour, with the mouth parts conspicuously yellow. Very soon after hatching the gut of larvae confined in a tube with moss was found to be coloured green, indicating that they had eaten the green parts of the moss. In general the larva resembles those of other members of the genus already described, but is more rugose. Other points which may be noted are as follows:

Head about equally long and broad, measured as for other species: longer than meso- or meta-thorax.

Mandible noticeably longer and more incurved than that of either *A. obscurus* or *A. sputator* (Text-fig. 1*d*). Apex long and sharply pointed, brown, the remainder yellow. Retinaculum longer than in the two species mentioned and distinctly curved backward towards the base. The sub-apical tooth is present as in other *Agriotes* larvae, but is long and narrow, extending as a kind of flange from the retinaculum nearly to the apex.

Nasale or clypeal process consisting of a single robust, somewhat pointed, tooth. Sub-nasal process projecting beyond the mouth cavity, margined with four or five sharply pointed teeth.

In the *antenna* the third or supplementary segment is of equal length to the conical ventral process. Both are longer than the 1st or 2nd segments, which are of about equal length.

Prothorax half as long again as either the meso- or meta-thorax.

Tergites of the body rugose, the rugae being principally transverse but running in all directions. *Setae* in general arranged as in other larvae of the genus, but there are six long, straight, out-standing ones near the apex of the 9th abdominal segment which are somewhat longer in proportion to the rest and stiffer than the corresponding setae of *A. obscurus* or *A. sputator* at this age.

The *spiracles* under a high magnification may be seen to have a distinct, though colourless, margin to the orifices, but they cannot at this stage be distinguished with certainty from those of the other two species.

The *9th abdominal segment* is gradually pointed but constricted before the apex at, and for at least a month after, hatching.

Cauda somewhat similar to that of *A. sputator* but rather more finely tapered. At hatching it is scarcely more coloured than the surrounding cuticle. *Sensory pits*, though present, are very shallow and their margins are not pigmented at first.

ATHOUS HAEMORRHOIDALIS, F.

This species is generally distributed and common throughout the country. The larvae are found in similar situations to those in which *A. obscurus* and *A. sputator* are found, but not in such great numbers. Consequently, though it feeds on the roots of similar plants, the damage done by it, as a species, is small in comparison. Perhaps the greatest damage done by the larva is to potatoes and to tomatoes in greenhouses. The length of the life history is long, probably as long as that of *A. obscurus*. Pupation occurs in August and the beetle emerges after a period of about three weeks. It remains in the soil during the winter, emerging therefrom in May.

Ovum.

An outline figure of an egg was given in Pt. I (p. 133, Fig. 5). The shape is very variable, though always rounded. It may be nearly spherical (·47 × ·42), bean-shaped in profile, or broadly ovoid. Average dimensions of four eggs ·51 × ·41 mm., the largest ·56 × ·43 mm. The shell is transparent, showing the milky-white contents within. Its surface when fresh is clearly granular as seen under a low power of the microscope, the granules being distributed thickly and evenly over the entire surface.

Thirty ova, most of them in a cluster, were found at $\frac{1}{4}$–$\frac{1}{2}$ inch below the surface of the soil on 12th July 1918. Many of them were observed to be advanced in development of the embryo and most hatched on 21–22 July, so that they must have been laid at the beginning of July at the latest.

Larva in First Instar.

Length at hatching from 1·5 mm. to 2·0 mm. Opaque white, head yellow and with some sign of yellow in the thoracic and 9th abdominal segments.

Head quadrilateral, a little broader than long. *Mandibles* stout, sharply pointed and brown at the apex, with a large, yellow, strongly-recurved retinaculum below the apex on the inner side. *Nasale* or clypeal process distinctly tridented; teeth sharply pointed. *Prothorax* as long as the two following segments taken together. On the dorsal surface it bears 4 setae, the posterior pair scarcely, if at all, longer than the anterior pair. The other segments of the thorax have only one pair of setae, at the posterior end. In the *abdominal segments* there is a transverse row of 4 long setae posteriorly, which are longer than the segments to which they belong. The length of the abdominal segments gradually increases from the 1st to the 8th.

The *9th abdominal segment* is broadest anteriorly and tapers to the posterior end, where it is narrowest. The space is nearly oval and is almost completely closed behind by the inner branches of the processes. This inner branch is tapered to a simple point and is not cuneiform, as in the older larva. The outer branch is yellowish and upturned but blunt at its apex. The two processes are yellowish. The posterior pair of marginal tubercles are alone visible at first, other two becoming visible later in the instar. The flattened disc of the dorsal surface is obvious even in newly-hatched larvae and the sagittal median furrow, with the transverse furrows, are visible in a suitable light beneath even a low magnification. The pseudopod, or anal papilla, is rather large.

Larva in Late Instars.

Length 20–22 mm., or according to Beling(2) 24 mm. with a width of 2·6 mm. Biconvex, deep yellow and strongly chitinized on the dorsal surface. Head, prothorax and 9th abdominal segment brownish yellow.

Head transverse, somewhat rounded at the sides. Dorsal surface considerably excavate in the occipital region and apex of the cephalic plate truncated. *Eyes* dark brown, situated posterior to the antennae on either side. A pair of moderately long setae are borne near each of the anterior angles of the head and a longer pair laterally a little posterior to the middle. There is also a longitudinal line of four setiferous follicles on each epicranial plate, midway between the middle and the lateral margin of the head: the most anterior of the setae arising therefrom is long, the remainder very short. *Antenna* longer than that of *Agriotes*, borne on a pale membranous base. The first and second segments brown, with apex of each pale. The third or dorsal supplementary segment is fine, linear and only about one-half the length of the second segment, which itself is about half the length of the first. The ventral process at the apex of the second segment is conical, short and colourless.

Mandible (Text-fig. 1*b*) stout, yellow at the base, dark brown and pointed at the apex, strongly curved inwards; with a rather long, slightly recurved retinaculum, inclined at an angle of about 45° to the apex. There is a penicillus at the base of the inner margin, composed of fine, somewhat wavy, yellow hairs.

Maxillary cardo more distinct than in *Agriotes*, flattened anteriorly at the point of articulation with the stipes and narrowed to a point posteriorly. Sides of the *stipes* nearly parallel and base almost straight and at right angles to them. *Maxillary palps* somewhat long, borne on white membranous palpigers which taper from their bases.

Galea with the first segment much rounded, especially on the outer side.

Lacinia triangular, pointed at the apex and densely clothed with long yellow hairs.

Nasale or clypeal process (Text-fig. 1*c*) brown, broad at the base, transverse, bearing three rather sharp teeth. The median tooth projects forwards, the two lateral ones outwards at an angle of 45°.

Prothorax nearly as long as the meso- and meta-thorax together, shallowly and sparingly punctured, almost smooth. Each succeeding segment to the 8th abdominal segment is progressively more densely punctured and the abdominal segments from 1st to 8th are dorsally

progressively more rugose, the first bearing only a few punctures and rugae. Anterior and posterior margins of the prothorax and posterior margins of the other two thoracic, as also the abdominal tergites 1–8, with border of longitudinal striations as in *Agriotes*. *Muscular impressions* of meso- and meta-thorax brown, somewhat raised, forming a slightly obtuse, rounded, angle near the antero-lateral margin. The lateral branch does not quite reach the middle of the segment and the transverse branch extends only half the distance to the medio-dorsal suture. In the first eight abdominal segments the transverse branch from either side meets its fellow at the medio-dorsal line and the longitudinal branch extends posteriorly to the striated border of the tergite. *Legs* rather short and of the same general type as those of *Agriotes*, bearing a few long setae on the inner side and a number of short brown spines arranged in rows on the remainder. Claws rather long, brown, sickle-shaped. Coxae almost globular, strongly chitinized on the inside but membranous on the outer side of each and allowing free movement to the first two segments of the leg within. There is a short oblique dark line in the chitin of the coxa pleurally, from which spring three short stiff spines on the meta-thorax and four on the meso-thorax, though the spines are absent from the prothorax. *Abdominal tergites* 1–8 with a transverse row of 6 long setae near the posterior margin on either side of the medio-dorsal line, and a shorter seta above each spiracle.

Pleurae chiefly membranous, but bearing an elongate sclerite of hard yellow chitin ventral to the spiracle on each. A single long seta is borne at the posterior end of this sclerite, corresponding to those of the tergite and sternite. *Abdominal sternites* 1–8 almost smooth, but with a few fine punctures, yellow and fairly strongly chitinized. Their shape is nearly square but with each postero-lateral margin excavate in proximity to a small, almost triangular sclerite of strong, yellow, chitin. This small sclerite bears a long tapered seta near its posterior margin. A pair of long setae are also situated on the posterior margin of the sternite, one near each lateral angle, and a pair of short fine setae between them. There is a short stiff seta near the anterior angle of the sternite on each side.

The thoracic *spiracles* are situated a little more ventrally than those of the abdominal segments, in the anterior of two isolated rounded sclerites lying between the tergite and sternite on either side of the meso-thorax. Those of the abdominal segments 1–8 are borne in the membrane of the epipleura, midway between the tergite and the elongate pleural sclerite. In shape they are elongate, with a median septum and are little broader anteriorly than posteriorly. Their margins are brown and bear

a large number of fine corrugations along their edge. The stigmatic scar is linear, placed transversely close to the anterior margin of the spiracle.

The *9th abdominal segment* (Plate XIV, fig. 2) is flattened on the dorsal surface and is bordered by a raised rim of chitin. It has a median longitudinal furrow, from which three principal tributary furrows branch obliquely forward, connecting with the lateral furrows. These extend in a somewhat indefinite line midway between the lateral margins of the disc and the central furrow. The remainder of the dorsal surface is punctured and furrowed irregularly. At the side of the segment, on the marginal rim, there are four large brownish tubercles, each bearing a long seta from its side, and ventral to these, below the rim, are three or four more similar but smaller tubercles. The shape of the disc is almost circular.

The space between the terminal processes is semicircular anteriorly, but posteriorly it is almost closed by the two converging inner branches of the processes, which are nearly cuneiform. The outer branch of each process terminates in a strong brown, upwardly curved hook, which itself bears a small accessory hook on its inner margin.

On either side of the segment from the anterior margin of the disc, a brownish raised line, resembling the transverse muscular impression of the other abdominal segments, is continued ventrally to the marginal border of striae which surrounds the sternite and pseudopod.

Pupa.

Length of male pupa in natural arched position 10 mm., expanded after fixation 13 mm.; breadth 3 mm. In general it resembles that of *Agriotes obscurus* and bears *spines* at the anterior and posterior angles of the pronotum as well as at the posterior end of the 9th abdominal segment. An additional pair is however borne by this species (and some other Elaterid pupae), one on either side of the median suture at the base of the pronotum. These project outwards and somewhat forwards and are shorter than those at the posterior angles. *Prothorax* both actually and relatively longer than that of *A. obscurus*, with its sides more parallel. *Metathorax* is also longer, bearing a somewhat wide longitudinal suture in its median line, which does not reach either to the anterior or posterior margin of the segment. *Antennae* reach just beyond the intermediate femora. A little below the apex of each antennal segment from the 3rd to the last there is a whorl of small tubercles. *Maxillary palps* rather long and incurved. Apices of *elytral cases* tapering to a fine point and bearing at the apex a short reflexed brownish hook.

The margins of both *tergites* and *sternites* of the abdomen are somewhat more produced than in *A. obscurus*. Dorsal surface minutely and shallowly punctate. Sternite of 7th abdominal segment produced in the form of a triangle, partially covering, and the apex reaching to the posterior margin of, the 8th segment. *Abdominal spiracles* situated in the pleurites, near their anterior margins, thoracic spiracles between the pro- and meso-thorax. In shape they are also like those of *A. obscurus*. as well as in position. The *terminal* pair of *spines* are somewhat short and bear on the inner margin of each a short sharp process in the male and female pupae examined. Sexual organs visible on the ventral surface of the 9th abdominal segment and approximately similar to those of *Agriotes*.

CORYMBITES CUPREUS, F.

This is a mountain-loving species and extends in suitable situations throughout temperate and central Europe to the Caucasus (du Buysson). The form *aeruginosus* appears to be merely a colour variety and is generally found where the typical form occurs. In Great Britain and Ireland it is widely distributed, but is common only in the higher-lying districts. In such localities the larva is commonly found in turf and under stones and, though no records of its harmfulness are known to the writer, it seems probable that minor damage may have been done, since the larva feeds in captivity on the roots of various plants. It is only fair, however, to point out that Xambeu(11) found them feeding on larvae of *Aphodius* and it is possible that both animal and plant food is taken. Other species of the genus are well-known pests of crops, principally in America(6).

The larva apparently moults twice in the year and eventually pupates in an earthen cell in the ground in July or August. It emerges from the pupal condition in about three weeks, but remains in the earth as a beetle during the winter.

Beling(2) has described the larva and pupa of this species under the name of its variety *aeruginosus* and distinguishes it(3) from the very similar larva of *C. pectinicornis* by the stronger punctuation and rugosity of the abdominal tergites.

Larva.

Length up to 25 mm., breadth across thorax 3·5. Colour above olive brown, with the sides, the ventral surface and usually the posterior margins of the segments yellow. The medio-dorsal suture and the membranous parts of the cuticle white.

Head broader than long, brown above, yellow beneath. Occipital

region somewhat excavate and the cephalic plate truncated behind. *Eyes* black. Four setiferous follicles in a line on each epicranial plate, as in *Athous haemorrhoidalis*, but the setae are fairly long. *Mandibles* short and broad, incurved, brown at the apex, with a penicillus at the base on the inner side. A strong tooth (retinaculum) of brownish colour is situated on the inner margin nearer the apex than the base. It is scarcely recurved but in position is inclined somewhat towards the apex. *Hypostome* wider in front than behind, with outer margins nearly parallel. There is an indication of a sub-galea at the base of the triangular lacinia, separated from the palpiger by a more or less distinct suture. *Nasale* or clypeal process composed of a single triangular tooth, rather long and pointed. Sub-nasal process apparently absent.

Prothorax as long as meso- and meta-thorax together, sparsely punctured with shallow punctures and having a few irregular furrows. Meso- and meta-thorax with muscular impressions as on abdominal segments, but shortened, the transverse branch reaching only half way to the median suture and the lateral even more reduced. *Coxae* somewhat similar to those of *Athous haemorrhoidalis*, with a similar oblique row of short spines arising from a linear cleft a little anterior and ventral to them.

Surface of *abdominal tergites* 1–8 sparingly but deeply punctured, chiefly in front. Punctures and rugae increase in number successively on each segment. Anterior setae consist of two short ones, one on either side of the median suture; the posterior row of five longer ones on either side of the median suture, arranged transversely. *Muscular impressions* brown, raised above the surface of the tergite, and together forming nearly a right angle; transverse branch somewhat sinuate and not quite reaching the median suture; lateral branch not quite reaching the posterior row of setae.

Pleurites elongate, narrowed in front and behind. Each bears two contiguous setae, a long and a short one, in line with the posterior row of tergal setae. Ventral to these and situated within the membranous part of the pleura (hypopleurite?) is another seta of medium length. *Sternites* quadrangular, almost smooth, bearing a longitudinal row of three setae along the lateral margin on each side. A single shorter seta is placed between the most posterior of these and the medio-ventral line.

Spiracles rather short, almost pyriform, broader in front than behind; situated within the membrane between tergite and pleurite. Their margins are brown, as also is the transverse scar placed almost immediately anterior to each. The cuticle anterior to the spiracle is corneous for a short distance only.

9th abdominal segment (Plate XIV, fig. 3) above deeply and fairly strongly punctured anteriorly. Disc with four principal furrows; the middle pair, situated one on either side of the middle of the segment, converging posteriorly but not meeting; the outer and longer pair nearly parallel to the lateral margins of the disc. A number of irregular tributary furrows join the principal ones at all parts of their length and sometimes connect them. Margin of the disc raised above its level anteriorly and at the sides in a rim running out into the recurved prong of the outer branch of the terminal process on either side. Inner branches of terminal processes pointed, with the apices all but meeting and enclosing the space between the processes. The space so enclosed is nearly oval. At each side of the disc, just ventral to the marginal rim, are three principal oval brown tubercles, from the side of each of which a long curved seta arises. A number of smaller brown setiferous tubercles are situated ventral to the three principal ones.

The anterior margin of the disc is continued down the sides in a slightly sinuous raised impression almost to the arched striated border separating the tergite from the sternite. A row of about twelve setae of various lengths forms a line at the edge of the striated border surrounding the pseudopod or anal tube.

Mr K. L. Henriksen of the University Zoological Museum, Copenhagen, has kindly given permission for use to be made of his generic table, originally published in the paper so often referred to here. Certain verbal alterations have been made and the table itself altered so as to include as far as possible the British genera of Elateridae, while excluding those unknown to this country. I would here tender my sincere thanks to Mr Henriksen.

Larvae of British Elateridae.

1.	Abdomen soft, whitish	*Cardiophorus*
	Abdomen with terga at least strongly chitinized	2
2.	Submentum triangular. Retinaculum absent	*Lacon*
	Submentum somewhat linear. Retinaculum present	3
3.	9th abdominal segment with a single tooth at the apex or simply rounded	4
	9th abdominal segment ending in two short processes	12
4.	9th abdominal segment simply rounded at the apex	5
	9th abdominal segment ending in a tooth	6
5.	Tergites punctulate. Head convex above	*Sericosomus*
	Tergites densely rugose transversally. Head flattened above	*Ludius*
6.	9th abdominal segment flattened above, angular at sides	*Melanotus*
	9th abdominal segment rounded above, conical	7
7.	Integument without deep punctures or rugae	8
	Integument coarsely punctured	10

8. 9th abdominal segment with transverse rows of large setiferous tubercles, without sensory pits 9
   9th abdominal segment without transverse rows of setiferous tubercles, with two sensory pits . . . . . *Agriotes*
9. 9th abdominal segment with 3 rows of setiferous tubercles . . . *Dolopius*
   9th abdominal segment with only 2 rows of setiferous tubercles *Adrastus limbatus*
10. Anal tube situated under the posterior third of 9th abdominal segment *Megapenthes*
    Anal tube situated under the anterior third of 9th abdominal segment . . 11
11. 9th abdominal segment narrowed conically from the base, with transverse rows of large setiferous tubercles . . . . . *Ischnodes*
    9th abdominal segment cylindrical at the base, without tubercles . . *Elater*
12. The two terminal processes simply bending inwards . . . . *Limonius*
    The two terminal processes each ending in 2 prongs 13
13. Inner prong of terminal process forming principal branch . . . *Hypnoidus*
    Outer prong forming principal branch or equal to the inner prong . . . 14
14. 9th abdominal segment with a more or less deep median furrow . . . 15
    9th abdominal segment without median furrow . . . . *Corymbites*
15. Nasale ending in a single tooth . . . . . *Corymbites aeneus*
    Nasale ending in 3 teeth 16
16. Space between the two terminal processes small with narrow aperture posteriorly 17
    Space between the two terminal processes large with wide aperture *Athous* (pars)
17. Terminal process with inner prong quite smooth and simple . . . *Campylus*
    Terminal process with inner prong very rough or projecting forward within the space . . . . . *Athous* (pars)

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EXPLANATION OF PLATES XIII AND XIV.

Fig. 1.

Larva of *Agriotes sputator*, L. *a*. Terminal segments. *b*. Cuticle of 9th abdominal segment between the sensory pits. Highly magnified. *c*. Cuticle of 8th abdominal segment along medio-dorsal suture. Highly magnified. *d*. Normal spiracle. *e*. Abnormal spiracle. *x*. Granulations of anterior margin of tergite.

Fig. 2.

Athous haemorrhoidalis, F. Dorsal surface of 9th abdominal segment of larva.

Fig. 3.

Corymbites cupreus, F. Same.

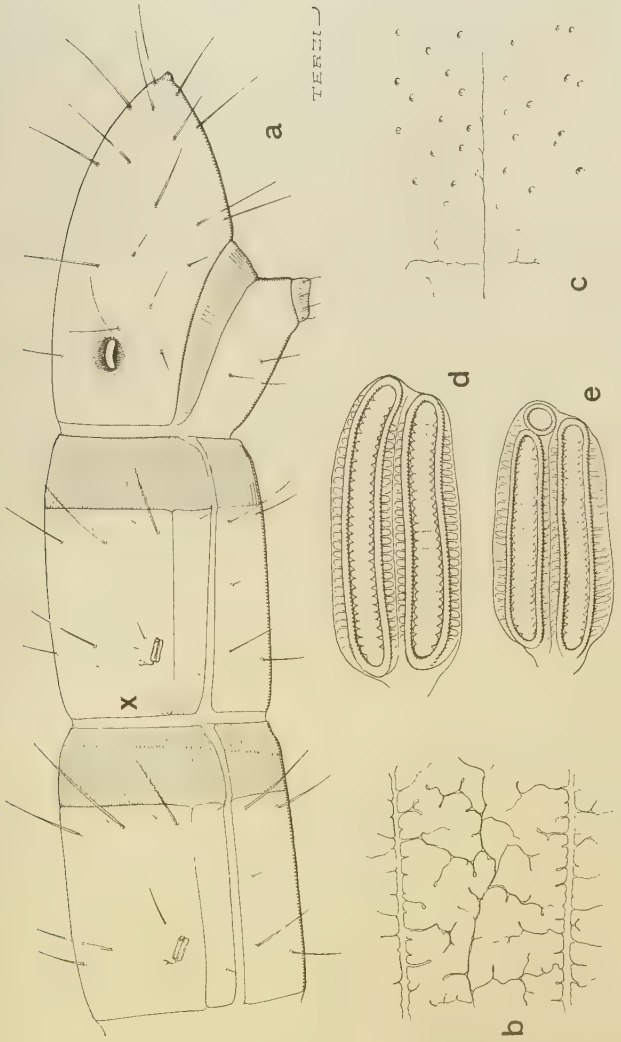


Fig. 1.

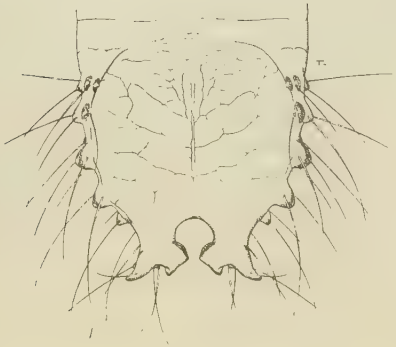


Fig. 2.

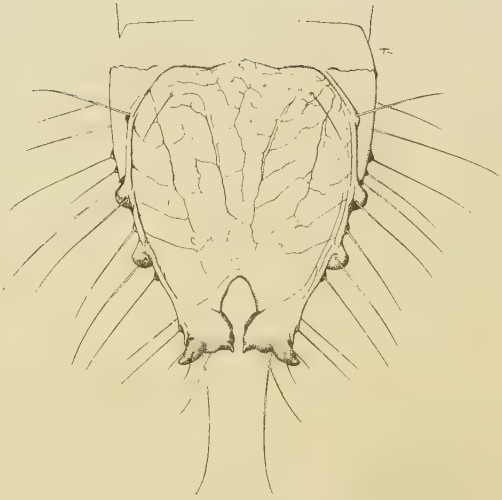


Fig. 3.

THE ACCURACY OF THE PLATING METHOD OF ESTIMATING THE DENSITY OF BACTERIAL POPULATIONS

WITH PARTICULAR REFERENCE TO THE USE OF THORNTON'S AGAR MEDIUM WITH SOIL SAMPLES

By R. A. FISHER, M.A., H. G. THORNTON, B.A., and W. A. MACKENZIE, B.Sc.

(*Rothamsted Experiment Station*)

(With 2 Text-figures)

1. Introduction

The accuracy of the estimates of bacterial density, in samples of soil, water, or other material, obtained by the plating method, is only one of many points which arise in the interpretation of bacterial counts. The full interpretation of such data would include a consideration of the divers species that occur on the culture media, and of the forms in which they exist in the soil. The partial or total exclusion of certain forms, such as anaerobes, that require special cultural conditions, must also be considered in a full examination of such data, for a single medium supplies, necessarily, but a single aspect, however comprehensive, of the bacterial flora of the soil. Questions too, as to what is to be considered as the unit of enumeration—the individual organism as it exists in the soil, or possibly groups of such organisms adhering to single particles of soil, and undetached by the processes of sampling and dilution—whatever their importance may be, are not the object of the present investigation.

For if all these inquiries could be answered with certainty and precision it would still remain to be discovered with what accuracy the numerical estimate of bacterial density, obtained from a single set of plates, represented the actual bacterial density in the sample, and in the material from which the sample was drawn.

The question of *accuracy*, therefore, unlike the other elements in the interpretation of bacterial count data, is primarily a statistical question

and may be thrown into the characteristic statistical form of the estimation of a population from a sample. Only in peculiarly favourable cases, however, as will be seen more clearly below, could we rely upon an *a priori* mathematical solution.

2. The Plating Method

The plate method of counting soil bacteria is an adaptation of the plate counting technique, developed by Koch in 1881, applied to the special conditions of soil bacteria.

The process in general consists in making a suspension of a known mass of soil in a known volume of salt solution, and in diluting this suspension to a known degree. The bacterial numbers in this diluted suspension are estimated by plating a known volume in a nutrient gel medium and counting the colonies that develope on the plate. An estimate of the bacterial numbers in the original soil is then made by a simple calculation, the mass of soil taken and the degree of dilution being known.

There are great variations in the details of the method as employed by various workers. These differences concern all the stages in the process and also the nature of the gel medium used in plating. An idea of the extent of this lack of standardisation may be gathered from a paper by Z. N. Wyant(16) in which a number of the variations in technique used by different workers has been collected from the literature.

As an example illustrating the process, however, the technique used at Rothamsted and employed by Cutler in the bacterial count work discussed below, will be described.

Ten grams of the soil sample are placed in 250 gm. of sterile saline solution and shaken for four minutes to obtain a suspension of the soil. 1 c.c. of this suspension is placed in 99 c.c. of sterile saline solution and shaken for one minute to ensure a uniform distribution of the contained organisms. 1 c.c. of this second dilution is placed in another 99 c.c. of saline and shaken for one minute.

Every cubic centimetre of this final dilution will then contain $\frac{1}{250000}$ grams of the original soil sample.

One c.c. of this dilution is then delivered into each of five petri dishes and mixed with an agar medium. After incubation the bacterial colonies on each plate are counted, and the mean of the five parallel counts taken. From this the bacterial numbers per gram of soil are estimated.

The bacterial numbers obtained by the plating method do not represent the total bacterial content of the soil. This is clear from the fact

that on no single medium will all the physiological groups of soil bacteria develope. In using this method, however, it is hoped to obtain a standard of bacterial density by which two or more soil samples can be compared. To obtain this result from the method a careful standardisation of the whole technique is essential, in order that those sources of error that cannot at present be eliminated, such as the failure of some organisms to develope on the plates, may be rendered so uniform as to affect the count in a constant manner.

This standardisation must comprise both (*a*) the manipulative portion of the technique involved in making the dilutions, and (*b*) the composition of the medium employed in plating.

In applying results obtained by the method it is necessary to have an estimate of its degree of accuracy, and in order to improve it, some knowledge must be obtained as to which stages in the process are the chief causes of the variation in results.

For the results of the plating method to have their highest possible accuracy, very severe conditions would have to be fulfilled. An imaginary experiment will perhaps serve to make the conditions clear.

If a 10 gm. sample of soil were diluted down to a dilution of 1 gm. in 250,000 c.c., enough material would be provided for $2\frac{1}{2}$ million plates. The result of such an experiment would be of the highest possible accuracy, if one could assume that

(I) Each plate offers the same facilities for development.

(II) The development of any organism is independent of other organisms present.

(III) Development results in only one visible colony.

Since in practice only a few plates are prepared, two additional conditions are involved in the sampling theory.

(IV) Each plate has an equal chance of receiving any organism.

(V) The organisms are distributed independently.

The fulfilment of the first, fourth and fifth conditions depends upon the perfection of the technique employed. The second and third conditions depend definitely on the nature of the organisms, and are only matters of technique in so far as this term may be employed for the choice or elaboration of a medium upon which the organisms, which it is desired to study, fulfil those conditions, and which excludes the interference of those which would fail to do so.

These conditions can to some extent be tested independently. Thus, in a short experiment, where a single batch of medium is used, it is to be expected that the medium in each plate will offer the same facilities

for development (Condition I). In a long experiment, however, where a number of different batches of medium are used, this will be the case only if the medium can be accurately reproduced, if, that is, different batches of medium, prepared independently, give significantly the same results. This reproducibility has been confirmed for Thornton's agar medium (Thornton, 1922(11)).

Again condition (IV) would fail if from any cause the dilution was carried out in an irregular manner. This may be tested directly by carrying through the whole dilution process independently with different portions of the same sample. The following experiment is an example of such a test.

Four portions of a sample of Barnfield soil, simultaneously analysed by four different workers (Aug. 14, 1921), gave the following counts:

Table I

	Portion			
Plate	A	B	C	D
1	26	28	31	37
2	30	33	26	32
3	30	32	28	32
4	29	26	32	30
5	32	27	31	26
Mean	29·4	29·2	29·6	31·4

The four sets of plates are indistinguishable from random samples from a single population. The variance estimated as from a single sample of 20 is 8·52, actually less than the mean value for the variance within each set, 9·15. An equivalent test is provided by the correlation between different plates of the same set; this is $-·089 \pm ·108$, negative and quite insignificant. In spite of the fact that the different plates of the same set agree very closely, the variation between the four means is quite insignificant.

Table II

	Portion			
Plate	I	II	III	IV
1	72	74	78	69
2	69	72	74	67
3	63	70	70	66
4	59	69	58	64
5	59	66	58	62
6	53	58	56	58
7	51	52	56	54
Mean	60·86	65·86	64·28	62·86

Equally close is the agreement between the sets of seven plates prepared from four parallel series of dilutions (June 22, 1922), shown in Table II. No trace of differentiation is observable, and the four sets must be regarded as random samples from a single population.

On certain occasions the same point is established by the analysis of simultaneous samples from the same field. An agreement in such cases shows the uniformity in bacterial density of the portion of the field sampled; it also serves to show that no significant differences are introduced by variations in the process of dilution. Thus four simultaneous samples from Broadbalk (Aug. 14, 1921) gave the following counts.

Table III

	Sample			
Plate	I	II	III	IV
1	38	45	43	27
2	32	40	34	41
3	·52	45	52	35
4	32	31	55	36
5	40	43	38	45
Mean	38·8	40·8	44·4	36·8

From the whole set of 20 the variance is 56·27, from the four sets of 5, 56·97, not a significantly greater value. The correlations between plates of the same group is $+ \cdot 014 \pm \cdot 108$, an insignificant positive value. By the most sensitive tests possible, no differentiation is observable.

There is thus reason to claim that the manipulative technique can be so efficiently standardised that no significant variations in it are detectable, having regard to the variance that occurs between the colony numbers developing on parallel plates from a single final dilution.

Our attention is thus drawn to this variance between parallel plates, which may be due solely to the chance distribution of organisms within the final dilution, or may in addition be influenced by the mutual interference between organisms on the plates, or by the failure of certain organisms to develope into single discrete colonies.

It is therefore necessary, in interpreting the results of the counting technique, to discover the relative importance of these influences, on the colony numbers, and on the variance between them. It is on the experimental evidence as to the actual nature of this variance between parallel plates that our further conclusions will be based.

Nevertheless, the two questions of the reproducibility of the medium and of the equivalence of results obtained by independent series of

dilutions made from a single sample, are here insisted upon, because failure in either of these two points would not necessarily affect the agreement between parallel platings, from the same final dilution, which is studied below.

3. The Poisson Series

It was shown by Poisson (1) in 1837, that if a large number of individuals, N, are each exposed independently to a very small risk of an event of which the probability of occurrence in any instance is p, then the number of occurrences, x, in any trial will be distributed according to a definite law, sometimes called the Law of Small Numbers. The distribution of x is found to depend on a single parameter

$$m = pN,$$

in such a way that the probability that the number of occurrences shall be x is given by the formula

$$e^{-m}\frac{m^x}{x!}.$$

It should be noted that x is always a whole number, while m may be fractional; the mean value of x is equal to m, and when m is large the distribution, except for its essential discontinuity, resembles a normal distribution, having its mean at m and the *variance* (the square of the standard deviation) also equal to m.

The importance of the Poisson series in modern statistics was brought out by "Student" (2) in 1907[1], in discussing the accuracy of counting yeast cells with the haemocytometer. Since the chance of any given yeast cell settling upon any given square of the haemocytometer is extremely small, while the number of cells is correspondingly great, "Student" arrived independently at the Poisson formula, as a theoretical result under technically perfect conditions. He was able to show that, in some instances, counts of 400 squares agreed with the theoretical

[1] The Poisson Series had been successfully applied by von Bortkiewicz to the annual number of deaths from horse-kick in a number of Prussian Army Corps (*10*). Miss Whitaker's criticism (*8*) of this application is entirely vitiated by her neglect of the variation of random samples.

H. Bateman (1910) (*9*) arrived at the formula for the Poisson Series, as the distribution of the number of α particles, emitted by a film of polonium, which strike a sensitised screen in successive equal intervals of time. The formula was used by Rutherford and Geiger to test the independence of simultaneous emissions. The distribution of 2608 counts shows a general agreement with expectation, though there are discrepancies not easily to be explained by chance. The observations are certainly not adequate, as these authors suggest, as "a method of testing the laws of probability."

distribution, and that when this is the case the accuracy of the count is known with precision and depends only on the number of cells counted[1].

The ideal conditions for bacterial counts made by the dilution method, are closely parallel to those found necessary in the case of the haemocytometer. The chief practical difference lies in the fact that instead of 400 squares with only a few yeast cells in each, we have some five plates with perhaps 200 colonies apiece. The agreement of the results with the theoretical distribution cannot, therefore, be demonstrated from a single count. Under ideal conditions the data would consist of a number of small samples from different Poisson series. For this reason as soon as it was suspected that this ideal condition might have been realized in practice, a special investigation of the nature of such samples was undertaken, owing to the importance of demonstrating the substantial fulfilment of the severe conditions laid down in the previous section.

4. Preliminary Reduction of Cutler's Data

When the question of the accuracy of the bacterial counting technique was discussed between the present authors in the spring of 1921, it was decided that the daily observations of bacterial numbers then being carried out at Rothamsted by Cutler would afford a valuable opportunity of studying the variance between parallel plates and its causes. In this choice our investigation was more than fortunate, for no other series of bacterial counts known to us, of which many have been examined, would have gone so far in clearing up the obscurities of the subject.

In conjunction with daily estimations of soil protozoa carried out at Rothamsted from July 1920, daily counts of bacteria were also made in the protozoological laboratory (Cutler(17)). The dilution technique used in this work has been described above. Plates were incubated at 18° C., and counted after five and seven days, the seven day counts only are considered here. Throughout the work the agar medium recently elaborated by Thornton(11) was used. The data thus supply an extensive test of this medium under routine conditions.

When the statistical examination of these data was commenced it was not anticipated that any clear relationship with the Poisson distribution would be obtained; the reduction was designed to determine empirically the relation between the mean bacterial number calculated from any set of plates, and the variability of that set about the mean. Knowing this relation, a probable error could be assigned to each value.

[1] Valuable tables of the Poisson Series have been prepared by H. E. Soper(7).

Two statistics were calculated from each set of plates. If x stand for the number of colonies on each plate, and n for the number of plates, the necessary statistics were:

the *mean* $$\bar{x} = \frac{1}{n} S(x),$$

and the *variance* $$v = \frac{1}{n-1} S(x - \bar{x})^2,$$

where S stands for summation.

The values of v, being the estimates of the variance from small samples, were inevitably affected by large sampling errors, which depended upon the number of plates. The whole body of four-plate sets was therefore divided into groups, according to the value of $\bar{x}$. Thus for the two groups of four-plate sets having a mean number of colonies 65–75 and 75–85, the following values of v were obtained:

Table IV

65–75		
Set No.	$\bar{x}$	v
29	69·75	65·58
33	73·50	27·00
51	68·75	312·25
60	71·50	401·67
128	73·75	60·91
164	72·75	146·25
227	67·50	27·67
241	68·75	8·91
249	67·25	7·58
263	73·25	112·58
272	72·75	52·91
330	70·00	55·33
Mean of 12		106·55
Mean of 10		56·47

Table V

75–85		
Set No.	$\bar{x}$	v
59	77·00	78·00
89	76·75	142·91
97	84·75	144·25
105	84·50	56·33
149	79·50	77·67
169	84·50	123·67
240	82·25	8·91
273	84·50	48·33
301	84·25	73·91
Mean		83·78

Two facts are apparent from these results (1) the variability of v is so great that accurate values are not obtained from the means of about 10 values; (2) the difficulty of estimating the variance for given values of $\bar{x}$ is still further increased by the occurrence of occasional very large values of v. The values of v in sets 51 and 60 in Table IV are much greater than the other 10 values in the same group. The values of the means obtained by excluding and including these high values are given at the foot of the table.

The first difficulty could be overcome by fitting to the actual values obtained a smooth curve representing the mean v for given $\bar{x}$; before

doing so, however, it was thought advisable to exclude as far as possible the exceptional large values. As a rough criterion it was decided to exclude those values which exceeded by more than threefold the mean value of the group. In the larger groups this criterion acted well; in the smaller groups, such as occurred for high and low values of x, it was necessarily inconclusive, even when account was taken of neighbouring groups. The curve fitting was therefore confined to the region in which the data appeared to be sufficiently abundant.

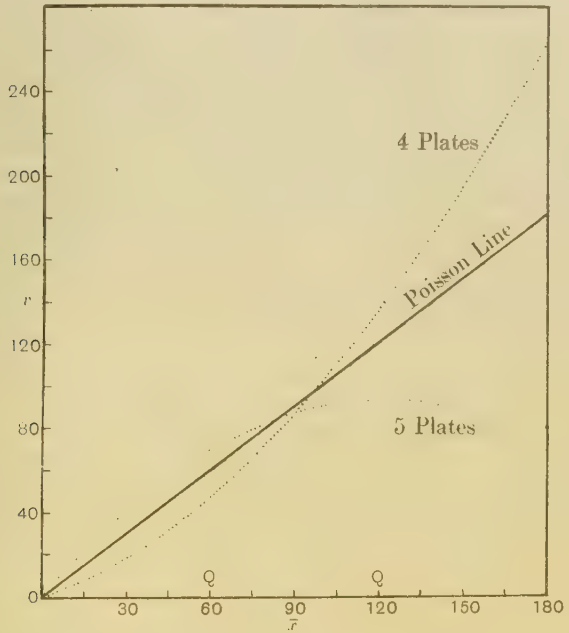


Fig. 1. Smooth curves fitted to Cutler's data.

Curves of the form $v = A\bar{x} + B\bar{x}^2$

(where A and B are two constants determined from the data) were fitted to the four-plate data from $\bar{x} = 0$ to $\bar{x} = 180$, and to the five-plate data from 0 to 160; the curves obtained are shown in Fig. 1.

The straight line, $v = \bar{x}$, represents the relation between the variance and the mean in the Poisson Series. The curves evidently tend to cling closely to this line, especially in the region (60–120) where the data are most abundant. The curves strongly suggested that the departures in

these data from the Poisson samples were not, as had been expected, *systematic*, but were due to the *sporadic* occurrence of exceptional sets; the curvature in the smooth curves being perhaps largely due to the crudity of the criterion employed in excluding the exceptions. This view impressed the authors with the necessity of studying the distribution of small random samples from the Poisson Series, with the double object of devising a valid criterion for the recognition of exceptions, and of testing accurately whether or not the remainder were in reality such random samples.

5. Small Samples of the Poisson Series

The study of small samples, essential as it is to the development of adequate statistical methods, has hitherto been practically confined to the normal curve and surface. The following investigation may serve to show, that by taking account of the fundamental properties of those statistics which are derived by the method of Maximum Likelihood, the sampling problems of even discontinuous distributions admit of material simplification.

In a sample from a Poisson Series, the chance of any observation having the value of x is

$$e^{-m}\frac{m^x}{x!},$$

where m is the parameter of the series.

Hence the chance of observing a given series of values $x_1, x_2 \ldots x_n$ is

$$\Delta f = e^{-nm}\frac{m^{n\bar{x}}}{x_1!\,x_2!\ldots x_n!}.$$

If we estimate m from such a sample by the method of maximum likelihood, we have

$$\frac{\partial}{\partial m}(\log \Delta f) = -n + \frac{n\bar{x}}{m} = 0,$$

so that $\bar{x}$ is the most likely value of m, and in consequence, as Fisher has recently shown(3), it may satisfy the criterion of sufficiency, in which case the distribution of any other statistic, for a given value of $\bar{x}$, must be independent of m.

That this is so may be proved directly; for

$$e^{-nm}\frac{m^{nx}}{x_1!\,x_2!\ldots x_n!}$$

may be put into the form

$$e^{-nm}\frac{(nm)^{n\bar{x}}}{(n\bar{x})!}\cdot\frac{(n\bar{x})!}{n^{n\bar{x}}\,x_1!\,x_2!\ldots x_n!},$$

the first factor represents the chance of obtaining a given value of $\bar{x}$, and the second, which does not involve m, gives the chance that the sample shall show any particular partition of the total, once the total is fixed. The distribution of any statistic which depends upon this partition, must therefore be independent of m, once $\bar{x}$ is fixed. The problem of the distribution of v is therefore susceptible of the great simplification, that we need only consider its distribution for given values of $\bar{x}$, and that this distribution is wholly independent of m.

The distribution of this, or any other, statistic, which depends upon a partition of an integer, must necessarily be discontinuous; when, however, $\bar{x}$ is large, even for small values of n, the number of possible values of v becomes sufficiently great for its distribution to be represented by a frequency curve. This procedure is the more advantageous in that, by the choice of a new statistic, which shall replace v, we can throw the distribution into a form independent of $\bar{x}$, whereas the actual partitions possible in the neighbourhood of equipartition, will necessarily change with the fractional part of $\bar{x}$.

The frequency with which any given partition of the total, $n\bar{x}$, occurs, is in fact the frequency with which any given series of values are obtained when the total is distributed at random into n cells, the expectation in each being $\bar{x}$. It is well known that when this is the case, the statistic

$$\chi^2 = \frac{1}{\bar{x}} S(x - \bar{x})^2 = (n - 1)\frac{v}{\bar{x}}$$

measures the departure of the sample from equipartition, being equivalent mathematically to Pearson's test of agreement between observation and expectation. The distribution of $\frac{1}{2}\chi^2$ is well represented by a smooth curve independent of $\bar{x}$ of the form (Pearson's Type 3)

$$df = \frac{1}{\left(\frac{n-3}{2}\right)!} t^{\frac{n-3}{2}} e^{-t} dt,$$

and the frequency with which χ^2 exceeds successive integral values, has been tabulated by Elderton (4, 1902 and 5, 1914) for values of n from 0 to 30.

We are therefore in a position to test whether the conditions which lead to the Poisson Series are in fact fulfilled in any given body of bacterial data for which the counts on individual plates are known; it is only necessary to calculate the above index of dispersion (χ^2) from each set of parallel plates, and to determine whether the distribution of this

index is or is not in accordance with the distribution predicted from Elderton's tables, when

$$\chi^2 = \frac{1}{\bar{x}} S\,(x - \bar{x})^2$$

and $$n' = n.$$

The statistic χ^2 thus supplies an index of dispersion for sets of parallel plates. If the bacterial counts conform to the Poisson distribution the average value of χ^2 will be one less than the number of plates. For sufficiently numerous sets of plates the agreement may be tested more exactly by the use of Elderton's Tables.

6. The χ^2 Index of Dispersion applied to Cutler's Data

The values of χ^2 obtained from the sets of four parallel plates, grouped according to the value of the mean, are shown in Table VI.

Table VI

χ^2

$\bar{x}$	·5	1·5	2·5	3·5	4·5	5·5	6·5	7·5	8·5	9·5	10·5	> 11	Total
20	2	—	—	—	—	—	—	—	—	—	—	—	2
30	1	—	—	—	—	—	—	—	—	—	—	—	1
40	2	—	2	—	2	1	—	—	—	—	—	—	7
50	—	1	2	5	2	1	1	—	—	—	—	—	12
60	5	3	2	—	—	—	—	2	—	—	—	12·3	13
70	3	2	4	—	1	—	1	—	—	—	—	13·6, 16·9	13
80	1	1·5	2·5	1	1	2	—	—	—	—	—	—	9
90	2	3	1	3	1	—	—	—	—	1	—	—	11
100	1	3	3·5	—	—	—	—	1	1	—	—	14·8, 24·5	11·5
110	—	2·5	1	1	—	—	—	—	—	—	—	—	4·5
120	2	—	—	1	—	—	1	—	—	—	—	15·1	5
130	—	—	1	·5	—	1	—	—	—	—	1	14·2	4·5
140	—	—	2	1·5	1	1	—	—	—	—	—	—	5·5
150	3	1	5	1	—	—	—	—	—	—	—	—	10
160	6	1	—	—	—	—	—	—	—	—	—	—	7
170	1	—	—	1	—	—	—	—	1	—	—	17·5	4
180	4	1	1	—	—	—	—	—	1	—	1	24·0, 13·9	10
190	1	—	·5	—	—	—	—	—	—	—	—	—	1·5
200	2	1	·5	—	—	—	—	—	—	1	—	15·8	5·5
210	1	1	—	1	—		—	—	—	—	—	—	3
220	—	—	1	—	—	—	—	1	—	—	—	12·2, 16·8	4
230	—	—	—	—	1	—	1	—	—	—	—	21·4	3
240	—	—	1	—	2	1	—	—	—	—	—	—	4
250	1	—	—	—	—	—	—	—	—	—	—	—	1
260	1	—	—	—	—	—	—	—	—	—	—	11·4	2
270	—	1	—	—	—	—	—	—	—	—	—	29·1	2
	39	22	30	16	11	6	5	4	3	2	2	16	156

No obvious relationships are observable between the value of χ^2 and that of $\bar{x}$. There is indeed an excess of the exceptionally large values of

χ^2 (> 11) among the higher values of $\bar{x}$, but this on investigation proved to be completely accounted for by the epidemic character of the occurrences of these large values, which we shall demonstrate below (see Fig. 2). The longest and most severe epidemic occurred during a period (Oct.–Dec.) when the bacterial numbers were generally high. Within this period no sensible association is apparent.

Confining attention therefore to the distribution of χ^2, irrespective of the mean number of colonies counted, it is clear that the sets with exceptionally large variations, which interfered with the preliminary reduction of the data, are now distinguishable as those with high values of χ^2. If the sets were random samples of Poisson Series, it appears from Elderton's Tables that only 3 per cent. of the observed values should exceed 9. It is clear that there is here a group which must be excluded in considering the agreement of the remainder with the theoretical distribution. If this were the only irregularity in the observed numbers we should therefore compare them with a theoretical series having the same total below 9. As it is there is also some irregularity visible at the beginning of the series, suggesting that there is also an excess of unduly small values of χ^2. For this reason we shall base our comparison on the total observed between 1 and 9, as is shown in Table VII.

Table VII

Comparison of observed and expected distribution of χ^2, 4-plate data.

χ^2	Expected m	Observed $m + x$	Difference x	$\frac{x^2}{m}$	
·5	24·97	39	+ 14·03		
1·5	28·76	22	− 6·76	1·589	
2·5	22·72	30	+ 7·28	2·333	
3·5	16·36	16	− ·36	·008	
4·5	11·27	11	− ·27	·006	·136
5·5	7·56	6	− 1·56	·322	
6·5	4·99	5	+ ·01	·000	
7·5	3·25	4	+ ·75	·173	·266
8·5	2·10	3	+ ·90	·386	
over 9	3·68	20	$\chi^2 = 4{\cdot}817,\ 4{\cdot}324$		
Total	125·66	156	P ·682, ·232		

Within the range from 1 to 9, the agreement of the observed with the expected values is striking. When tested in eight groups, the probability of obtaining a worse fit by chance from perfectly normal data is ·682,

and even when grouped in the most unfavourable manner, by throwing together consecutive positive and negative residuals, a method suggested by Mr Udny Yule, the probability is still ·232. There is therefore no significant deviation of those values from expectation.

Of those above 9, we may anticipate that some three or four will be normal values and the remainder exceptions. It is of course impossible to separate these with absolute certainty. In discussing the evidence for epidemics we shall assume that the four values below 11 are normal and that the remainder are exceptions. When, however, the fact of the epidemic incidence of those exceptional values is taken into account, it appears that the two between 10 and 11 are among the relatively few "normal" sets occurring in an epidemic period and are therefore probably exceptions, while the two between 9 and 10, and possibly also the value at 11·4, are for the same reason probably normal.

It is thus possible to separate this class of exceptions from the remaining data with some degree of certainty and to study them individually, but this is not possible for the exceptionally invariable sets. All that we can do here is to show that the evidence for their real existence is stronger than appears in Table VII. If we subdivide the region of the first two groups of that table somewhat more closely we obtain

Table VIII.

χ	Expected	Observed
0		
	11·82	21
·75		
	9·97	12
·95		
	12·56	17
1·15		
	14·15	9
1·35		

the excess of numbers is most clearly marked in the group of smallest values, and is possibly though not certainly confined to the region.

These conclusions are independently confirmed by the sets of five parallel plates. In Table IX is shown a comparison of the observed distribution with that expected, on the basis of the total observed between 2 and 11.

The agreement with expectation in the range from 2 to 11 is perfectly satisfactory; when tested in the 9 unit groups, the possibility of obtaining

a worse fit by chance from normal data is ·765. Grouping together the consecutive positive and negative errors, it only falls to ·571. There is again no significant deviation of the distribution in this range from expectation.

Table IX

Comparison of observed and expected distribution of χ^2, 5-plate data

χ^2	Expected m	Observed $m+x$	Difference x	$\frac{x^2}{m}$	
·5	10·94	25	+14·06		
1·5	21·10	27	+ 5·90		
2·5	21·58	24	+ 2·92	·271	·402
3·5	18·41	20	+ 1·59	·137	
4·5	14·39	12	− 2·39	·397	
5·5	10·69	11	+ ·31	·009	·146
6·5	7·67	9	+ 1·33	·231	
7·5	5·37	5	− ·37	·025	1·826
8·5	3·70	0	− 3·70	3·700	
9·5	2·51	3	+ ·49	·096	·157
10·5	1·68	2	+ ·32	·061	
over 11	3·22	18		χ^2 = 4·927, 2·938	
Total	121·26	156		P = ·765, ·571	

Of the values above 11, three lie between 12 and 13, and in discussing the evidence for epidemics we shall assume that these are normal sets, and that all those above 13 are exceptions. When we take the evidence of epidemic incidence into account, it is found that the only four sets above 13 which might reasonably be considered normal all occur in epidemic periods, and that the same is true of one out of the three between 12 and 13. This therefore (No. 160, see Fig. 2) is probably also an exception.

The conclusions to be drawn from the 4-plate and from the 5-plate data, thus confirm each other at every point. In both groups the sets having exceptionally high variability may be identified in almost every case with certainty. The majority of both groups, about 124 of the 4-plate sets, and about 117 of the 5-plate sets, are evidently true samples of the Poisson Series. Both groups show an excess of cases of small variability, but it is not possible to specify the actual sets affected by this; it is evident that this cause, like that which produces high variability, is sporadic and not systematic in its action; it affects a certain number of sets in a definite manner, leaving the majority unaffected. This effect, whatever be its nature, is more clearly brought out in the

5-plate than in the 4-plate sets, possibly because the sets of five plates make possible a closer scrutiny into the exactitude of the agreement between the observed sets, and samples from a Poisson Series.

For the same reason the 50 sets of three plates cannot be expected to provide much additional information. The seven exceptionally high values stand out perfectly clearly; the lowest is 9·2, a value which would be exceeded by only one normal sample (of 3) in 100. The next highest values 5·4 and 6·4, would not be suspect save for their occurrence in December; they will be treated as normal.

Since the 3-plate sets are relatively scanty, we can best test their agreement with theory by dividing the theoretical distribution of 43 values at its quintiles, so that the expectation is the same in each group. We then have

Table X. Sets of three plates

$\chi^2 = 1{\cdot}77 \qquad P = {\cdot}775$

χ^2	Expected m	Observed $m+x$	x^2
0	8·6	8	·36
·4464	8·6	6	6·76
1·0216	8·6	11	5·76
1·8326	8·6	8	·36
3·2190	8·6	10	1·96
Total	43	43	15·20

The agreement with expectation is excellent, and the sets of three plates bear out the conclusions derived from the sets of four and five plates, save that here there is no visible excess of low values of χ^2.

It appears therefore that out of the 362 sets of plates examined the majority represent true samples from the Poisson Series, such as would be the case if the biological and technical difficulties of the bacterial count method as applied to soil had been completely surmounted. Forty sets, which can be identified almost with certainty, are affected by some cause or causes which greatly increase the variability between the plates, while probably a smaller number, including apparently none of the 3-plate sets, are affected by a second cause of error, which reduces the variability between the plates.

7. The exceptionally Variable Sets in Cutler's Data

The records of the exceptionally variable sets of plates which occurred in Cutler's data, when identified by the method of the preceeding section, were studied individually with a view to gaining light upon the cause of their occurrence. As it is not necessary to reproduce the whole of the statistical tests which were applied, we shall confine ourselves to the main facts which emerged, and which served to justify the previous conclusions, as well as to indicate the nature of the disturbing cause.

The following facts appear to be unquestionable:—

(1) The proportion of exceptionally variable sets is the same for the sets of three, four and five plates in each portion of the total period.

(2) The proportion of exceptionally variable sets varies greatly at different periods, the exceptions occurring in well marked epidemics.

The evidence for these statements may be put in the form of a triple contingency table (see Fig. 2)

Table XI

	Excessively variable				Not excessively variable				Total				
Period	5	4	3	Total	5	4	3	Total	5	4	3	Total	χ^2
1	1	—	1	2	9	9	9	27	10	9	10	29	·967
2	3	2	1	6	7	12	6	25	10	14	7	31	1·728
3	—	—	1	1	12	18	4	34	12	18	5	35	6·176
		(10)		(14)		(11)		(20)					
4	3	9	1	13	5	12	4	21	8	21	5	34	·818
	(6)	(5)		(12)	(6)	(14)		(24)					
5	5	4	1	10	7	15	4	26	12	19	5	36	1·733
6	—	—	—	—	19	18	—	37	19	18	—	37	—
7	—	—	—	—	22	13	1	36	22	13	1	36	—
8	—	—	1	1	20	11	5	36	20	11	6	37	5·310
9	1	—	—	1	17	12	6	35	18	12	6	36	1·029
10	2	1	1	4	23	20	4	47	25	21	5	51	1·299
	(16)	(18)		(41)	(140)	(138)		(321)					
Total	15	16	7	38	141	140	43	324	156	156	50	362	19·060

in which the whole of the 362 observations are divided,

(1) according to the number of plates observed,

(2) in ten periods of time of alternately 36 and 37 days, into which the year was divided,

(3) according as they are judged to be exceptionally variable, or not, solely upon the evidence of the χ^2 index. The subdivision which would be made taking also into account the evidence for epidemics is shown in brackets, but in discussing the evidence for epidemics these modifications are ignored.

To test the first point, each line of Table XI is treated as a 2×3 contingency table, and the value of χ^2 calculated from it. It has been shown (Fisher, 1922(6)) that as in such a table there are two degrees of freedom, χ^2 will be distributed, if there is no association, as in Elderton's Tables when $n' = 3$. To show that at no period is there significant association, the values of χ^2 for the 10 periods are added, and the resulting quantity should be distributed as in Elderton's Tables when $n' = 21$. Since in two consecutive periods no exceptionally variable sets occurred, these periods have been omitted, and n' is taken to be 17. It will be seen from the table that all the values of χ^2 are less than 2, except in two periods in which only a single exceptionally variable set occurred. Such cases are evidently beyond the range of effective application of the χ^2 test, but even including these high values, $P = \cdot 266$, and therefore there is no significant departure from the rule that sets of three, four and five plates show equal proportions of exceptions in all sections of the period of observations.

This fact confirms the justness of the criterion by which the exceptions have been identified, for any error in the method of identification would naturally show itself in the proportion of cases regarded as exceptions; in the second place it indicates that the cause of exceptional variability is not connected with the causes which lead to the rejection of individual plates (contamination, development of fungi or overgrowth by *B. dendroides*), and in the third place it shows that the exceptions are not caused by the exceptional deviation of a single plate, for in this case the proportion of 5-plate sets would necessarily be highest. The third conclusion is borne out by an examination of the numbers counted on individual plates, and both it and the second conclusion are more decisively drawn from the contingency table by ignoring the period of occurrences.

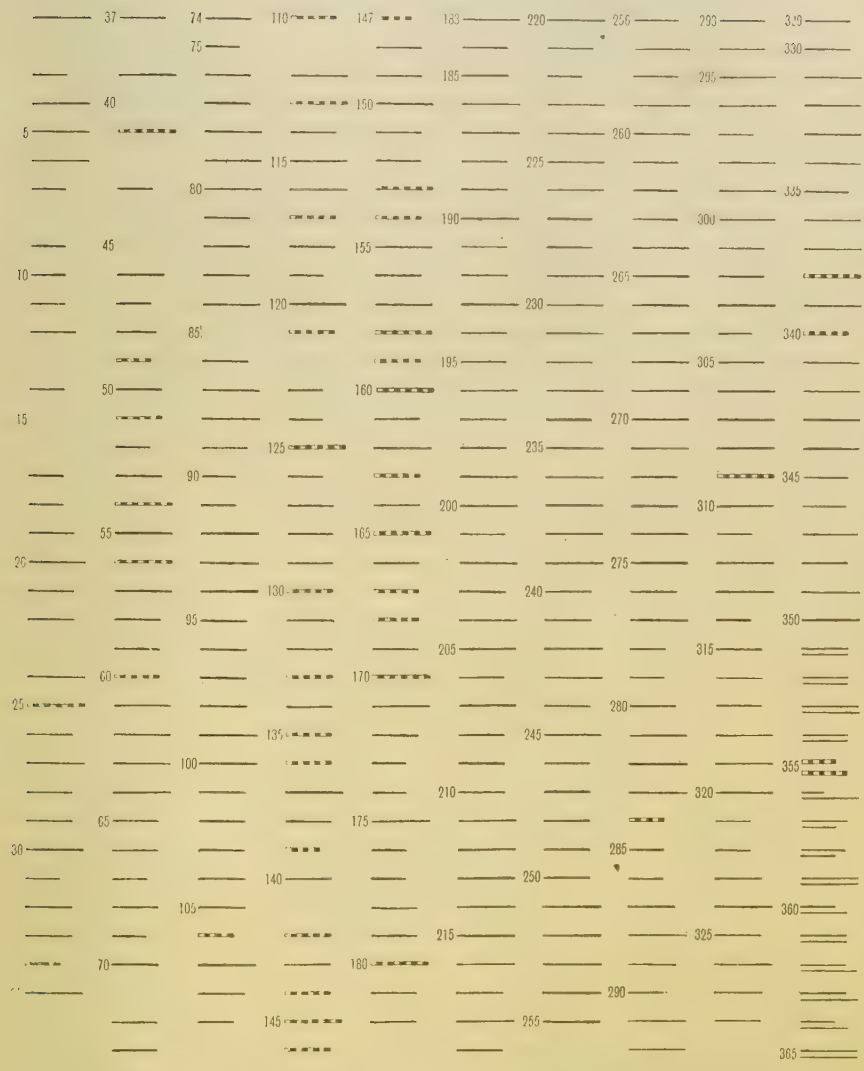
Table XII

No. of plates	5	4	3	Total
Exceptionally variable ...	16	18	7	41
Not exceptionally variable ...	140	138	43	321
Total	156	156	50	362

The numbers in the smaller groups are here sufficient to make a satisfactory test, and the value of P, ·739, shows distinctly that there is

Cutler's Data

Sets of Plates for each day of the Year.



EXCEPTIONALLY VARIABLE

NOT EXCEPTIONALLY VARIABLE

5 Plate

4

3

Fig. 2.

no significant difference in the proportion of exceptions between the several groups of observations.

Similarly the distribution of the exceptions in time, in which we have shown the different groups to agree, may be best shown by taking the totals, irrespective of the number of plates in each set. If this is done we have a 2×10 contingency table, of which the value of χ^2 proves to be 57·826.

Since $n' = 10$, the chance of such a distribution occurring under conditions of random occurrence in time is about 4×10^{-9}. It is indeed obvious from inspection of Fig. 2 that the exceptional values occur in groups together, although perfectly normal values continue to occur throughout the worst of these epidemics. During the first outbreak seven exceptions occurred with 14 normal values among them; the second epidemic period was more prolonged and included 27 exceptions and 46 normal values. In the second half year of the experiment only six exceptions occurred, of these two occurred on the same day (355) during the last fortnight, when duplicates were taken, and two others, 338 and 340, were but two days apart.

Bearing these points in mind, we have no hesitation in concluding, on purely statistical evidence, that the exceptionally variable sets of platings were due to two causes:—(*a*) a predisposing cause which is at work throughout the epidemic period, and (*b*) some additional circumstance, in the absence of which the counts obtained will still be normal.

8. Special Organisms which affect the Number of Colonies developing

In the daily counts above considered, a uniform technique was followed throughout, and fresh batches of medium were made up at frequent intervals. It is conceivable that occasional differences in plating technique, in the medium, or in counting the plates may by chance have occurred on certain days. It is however most unlikely that any such differences can have extended over the long periods covered by the epidemics of high variance, without the fact being noticed. In seeking a predisposing cause of variance, covering these periods, therefore, one's attention is naturally drawn to possible changes in the soil itself or in its population.

It is known that certain micro-organisms, when growing on the medium, exert an inhibitory action on the development of colonies by other forms. The appearance of such an organism in the soil population, during certain periods, might therefore give rise to periods of higher

variation between parallel plates, for unless present in very large numbers it would not appear on all the plates or even in every batch of five plates.

An example of high variation between parallel plates, that was actually traced to such an organism, is given to illustrate this cause of inaccuracy.

The soil used in this case was from the Leeds Experimental Farm, and had received a treatment of naphthalene. Thirty parallel platings of this soil were made on Thornton's agar. The counts of colonies on these plates are given in Table XIII.

Table XIII

Parallel plates of Leeds soil

Plate No.	Number of colonies	Plate No.	Number of colonies
1	*240*	16	126
2	*209*	17	126
3	*177*	18	126
4	*158*	19	121
5	157	20	120
6	*154*	21	119
7	151	22	118
8	137	23	117
9	136	24	114
10	132	25	113
11	131	26	109
12	131	27	99
13	130	28	*91*
14	128	29	*91*
15	127	30	*87*

χ^2 Index. Whole series = 230·17
Minus the italicised plates = 27·81

It will be seen that the variation between parallel plates in the whole series is excessive. In examining the plates, some were found to contain an organism forming a growth between the agar and the bottom of the dish. This organism occurred on the plates italicised in Table XIII. It is a motile organism and apparently spreads in the water film underlying the agar. On plates 28, 29 and 30, the growth of this organism was sheet-like and from the low counts obtained it would appear that its growth has reduced colony development. On plates 1, 2, 3, 4 and 6, it has produced a number of separate colonies underlying the agar. These colonies were probably produced by individuals which had multiplied and migrated along the bottom of the dish after the agar had set,

but could not be separated from other colonies in counting the plate. The counts on these plates are therefore excessive. The presence of this organism on the bottom of the plates has thus produced an abnormal variation in the whole series. It will be seen that, if plates on which it occurs are ignored, the χ^2 index for the remaining 22 plates falls within the expectation of random sampling.

A pure culture of this organism was obtained and a plating from a sample of Rothamsted soil was made, a small loopful of suspension of the organism being added to the first dilution flask. Table XIV, Series A, shows the colonies developing on six parallel plates of the soil thus treated, compared with a control series of plates of the same soil not inoculated, Series B, which were made at the same time.

Table XIV

Effect of Leeds soil organism on colony development from suspension of Rothamsted soil

Series A. Suspension inoculated			Series B. Control	
Plate No.	Number of colonies	Area of bottom spreading	Plate No.	Number of colonies
1	85	nil	1	95
2	79	nil	2	90
3	78	nil	3	86
4	70	nil	4	85
5	58	nil	5	85
6	60	2·25 sq. cms.	6	82
7	56	7·75 ,,	7	81
8	45	27·0 ,,	8	77
9	41	54·5 ,,	9	73
10	39	56·5 ,,		
χ^2 Index, Plates 1 to 5 = 5·86 Plates 1 to 10 = 40·01			χ^2 Index = 1·89	

In this case the organism formed a spreading growth over the bottom. The area of this spreading growth, where it occurred, was measured and is shown in Table XIV. It will be seen that the reduction in colony development is clearly related to the amount of spreading growth. In this series of plates it is also evident that the variation is greatly increased by the occurrence of the organism on certain of the plates.

From an abnormally variable series of plates of Rothamsted soil a second organism has been isolated, whose frequent habit it is to spread on the under surface of the agar, and which has a similar inhibitory action on the development of other colonies. Table XV shows two sets

of plates of a suspension of Rothamsted soil, one set of which was inoculated with this organism. The reduction of, and increased variation in colony numbers are again well seen.

Table XV

Effect of toxic organism from Rothamsted soil on colony development from a soil suspension

Series A Plates inoculated		Series B Control	
Plate No.	Number of colonies	Plate No.	Number of colonies
1	192	1	179
2	168	2	171
3	147	3	168
4	130	4	150
5	127	5	150
6	113		
Mean 146·1 χ^2 Index = 29·47		Mean 163·6 χ^2 Index = 4·17	

It is of course impossible to decide, with certainty, from a simple record of colony numbers, whether the presence in the soil of some such organism was the cause of the epidemics of variable plate-sets in Cutler's series. However, the above two cases of high variance between parallel plates, which have been traced to the presence of definite organisms, show that this factor, though apparently of infrequent occurrence, is capable of causing a disturbance in the colony numbers of precisely the kind actually observed. It is important to notice that this, probably like all other causes, that produce a sensible departure from the Poisson Series, seriously disturbs the mean value.

9. The Occurrence of Subnormal Variation

It has been shown that in a small proportion (about 34 cases) of Cutler's data, the variation between parallel plates has been apparently lowered by some disturbing agency. The same phenomenon in a much aggravated form appears in Owen's data (section 10), and has from time to time occurred in Thornton's work. For example the 20 plates shown in Table I display an unduly low variation, and though this fact does not detract from the value of the data in proving the equivalence of parallel dilutions, it does throw suspicion on the value of the mean as an estimate of bacterial density. A similar depression appears in Table XIV, Series B.

Unlike the excessively variable sets, the sets with subnormal variance cannot be identified individually in Cutler's data, and we have therefore less evidence upon which to put forward a biological explanation of the phenomenon; certain facts, however, concerning observations made in the course of 1921, suggest that additional precautions in the preparation of the medium, may be effective in eliminating the disturbing cause.

The additional data were accumulated in the Bacteriological Department[1] in the summer and autumn of 1921 in the course of some work on the relationship of bacterial numbers to nitrate content in the field soil. In each of these experiments a series of some 45 samples of soil were taken from a plot 9 by 15 feet in area and the bacterial numbers in each sample estimated by the plate method using Thornton's agar medium. The first experiment was carried out with the dunged plot in Barnfield. The technique used was similar to that employed in Cutler's work, five parallel platings being made of each sample and the colonies counted after an incubation of seven days at 20° C.

Of the 33 sets available, three show excessive variance, the remainder are distributed as in Table XVI.

Table XVI

$\chi^2 = 3{\cdot}08$ $P = {\cdot}381$

χ^2	5-plate	4-plate	3-plate	Total	Expected	x^2/m
·5	—	—	1	1	9·78	·79
1·5	4	2	—	6		
2·5	2	—	—	2	9·39	·21
3·5	4	2	—	6		
4·5	4	—	1	5	5·56	·37
5·5	—	2	—	2		
6·5	3	1	—	4		
7·5	3	—	—	3	5·06	1·71
8·5	1	—	—	1		
Total	21	7	2	30		3·08

It will be seen that these agree well with the Poisson Series, and show no sign of subnormal variation.

A second experiment was carried out at Kingsthorpe Hall, Northampton. The soil is here of a markedly different type from the heavy Rothamsted soil, being a light ferruginous loam. In this experiment the technique was varied in that the colonies on each plate were counted twice, after seven and twelve days' incubation. It will be sufficient to compare the observed and expected values of the total, $S(\chi^2)$, for different groups of plates.

[1] The authors wish to acknowledge their indebtedness for the assistance rendered by other Departments at Rothamsted in this work.

Table XVII

Number of plates per set	Medium	After 7 days		After 12 days	
		Expected	Observed	Expected	Observed
4	A	18	13·85	24	27·31
5	A	152	109·33	144	133·96
9	A	8	1·96	8	8·73
Total	A	178	125·14	176	170·00
20	B	19	19·45	19	25·34

In all these groups where medium A is used the variance is distinctly subnormal after 7 days, but is apparently normal after 12 days. With medium B, the variance is normal at both counts. Now the sets of 9 and of 20 plates were parallel dilutions of the same sample, and the mean count from medium A was only 75 per cent. of that obtained on medium B. The abnormality of medium A was afterwards traced to the temperature at which it was filtered, a technical detail which has an important bearing on the ability of the medium to support bacterial growth (Thornton, 1922(11)).

In the comparison given by Thornton(11) of the two batches of medium, identical save that one was filtered at 50° C. and the other at 100° C., 10 plates being prepared from each, the former gave a mean count 79 per cent. of the latter; in this case also the defective medium showed subnormal variance giving a value $\chi^2 = 3{\cdot}2$ (after eight days), whereas the normal medium gave a value 10·3. The former would only occur once in 22 trials by chance, and therefore represents clearly a subnormal condition.

Whatever the biological explanation of subnormal variance may be, it is therefore sometimes indicative of a serious error in the value of the mean. In this respect it is a danger signal which cannot be disregarded. When a set of plates shows excessive variability no one will be tempted to lay too much stress upon their mean; it is obvious in such cases that there is a large probable error, and it has been seen (Section 8), that there will usually be also a considerable systematic error in such cases. A set of plates with abnormally low variance on the other hand, may appear to be particularly good data, although, as we have just seen, this type of abnormality is also indicative of large systematic errors. It is therefore of practical importance that such departures from the Poisson distribution should be detected, whenever they occur. Since subnormal

variation cannot be detected with certainty in a small set of plates, we recommend that occasional sets of 10 or 20 plates should be prepared from time to time, and that if necessary every batch of medium prepared should be tested in this way, the colonies being counted after seven days.

10. The χ^2 Index of Variability applied to other Bacterial Count Data

It has been shown by the use of the χ^2 index of variability, that the great bulk of Cutler's data on soil bacteria appears to be true samples from the Poisson Series, and that therefore the accuracy of these results is known with precision; also that, by the same method, a small proportion of exceptions may be detected in which some definite disturbing cause has interfered with the accuracy of the results. It is therefore desirable to apply the same test to other sufficiently extensive bodies of material, in order to ascertain if, by other methods, a similar degree of accuracy can be obtained, and failing that, if further light can be thrown on the problems of the dilution method. Data from four sources have been examined in this way.

(A) Buddin's counts of soil bacteria at Rothamsted, using a gelatine medium.

(B) Counts of soil bacteria published by Engberding (1909 (12)).

(C) Breed and Stocking's tests of the accuracy of counting *B. coli* in milk (1920 (13)).

(D) W. Owen's bacterial counts in sugar refinery products (1914 (14)).

In the aggregate we have tested over 1000 sets of parallel plates; owing to the bulk of the total examined it is possible that a small proportion of arithmetical errors has been included, although the application of the method is much more expeditious than that of the preliminary investigation of Cutler's data. Only the obvious and unquestionable features of each body of data will be dealt with.

(A) *Buddin's data*

A very large number of bacterial counts were made at Rothamsted by W. Buddin, to whom we are indebted for permission to make use of these data. The actual plate counts, though not published, formed the basis of bacterial number estimations used in Buddin's work on the effect of antiseptics on soil (15).

The platings in this work were made on a nutrient gelatine having the following composition:—Witte's peptone 40 grams, Lemco 20 grams, NaCl 20 grams, gelatine 480 grams, distilled water 4000 c.c.

The counts therefore supply an example of the degree of accuracy obtained with a gelatine medium, where a considerable source of variance is produced by the occurrence of liquefying organisms on the plates.

From the mass of data available, 100 sets of triplicate platings were extracted. The expected and observed values of χ^2 in this series are shown in Table XVIII.

Table XVIII

χ^2	Expected	Observed	Difference
·5	39·3	25·5	− 13·8
1·5	23·9	26	+ 2·1
2·5	14·5	12	− 2·5
3·5	8·8	10·5	+ 1·7
4·5	5·3	6	+ ·7
5·5	3·2	4	+ ·8
6·5	2·0	3	+ 1·0
7·5	1·2	4	+ 2·8
over 8	1·8	9	+ 7·2
Mean	2·0	3·04	

There is a marked deficiency below 1, and an increasing excess above 3. No distinct class of exceptionally high values can be detected, only three values exceed 10, and none exceed 15. The causes of additional variability probably affect all observations in some degree, and are therefore systematic rather than sporadic. The mean variance is about 50 per cent. in excess of that due to random sampling. As in Cutler's 3-plate data the departure from expectation is best shown by dividing the distribution at the quintiles as in Table XIX.

Table XIX

$\chi^2 = 17{\cdot}4$ $\quad P = {\cdot}0017$

χ^2	Expected m	Observed $m + x$	x^2
0	20	12	64
·4464	20	15	25
1·0126	20	23	9
1·8326	20	15	25
3·2190	20	35	225
Total	100	100	348

Such a departure from expectation would occur by chance but once in 600 tests; it is therefore clearly significant. The technique used here did not therefore give results of such accuracy that the variance between parallel plates could approximate to the Poisson Series.

(B) *The data of Engberding* (12)

The parallel platings given by this author were made to test various points connected with the plate method of counting soil bacteria. Some of the sets of platings were made on a variety of gelatine and agar media, as a test of these. The majority, however, were poured on an agar medium, containing "Nahrstoff-Heyden," that was considered by the author to be the best of the media tested.

Engberding gives 24 sets of plates; of these, 14 are of six plates each, six of five plates, three of four plates and one of nine plates. Nearly all the sets show excessive variability; only three values out of the 24 are below the expected average for the corresponding number of plates. The total of the 24 values is 5·36 times the expected total. No further test is necessary; random sampling must be regarded as one of the smaller causes of variation in these data.

(C) *The data of Breed and Stocking* (13)

We next come to a very thorough attempt made by Breed and Stocking to test and improve the methods used in the bacterial analysis of milk. The medium used in the platings here considered had the following composition:—"Difco" peptone 1 per cent., lactose 1 per cent., "Lemco" ·3 per cent., air dried agar 1·5 per cent. A single batch of medium was used throughout each experiment, so that ability to reproduce the medium, is not here tested. Parallel samples of normal milk, and of milk inoculated with *B. coli*, were analysed by different analysts and at different stations. Two series of these records have been examined by comparing the different plates of each separate analysis. Each series yielded 132 sets of three numbers, the duplicate counts of the same set of plates being reckoned as two. If the duplicate counts had closely agreed, this would tend to give us a bad fit between observation and expectation, to the extent of doubling χ^2. Though the agreement is not sufficiently great to have this effect, the tendency is to be borne in mind.

The expected and observed distributions are shown in Table XX.

As with Buddin's data, though to a less extent, there is a small systematic excess of the larger values; the mean variance in series B is about 30 per cent. in excess of expectation, while in series C it is only

about 20 per cent. Series B also shows certain other irregularities and possibly the occurrence of sporadic causes of variation. Series C, which represents the final perfection of the technique employed, shows no excessively variable sets of plates.

Table XX

χ^2	Expected	Series "B"	Series "C"
·5	51·9	46	43
1·5	31·5	35·5	30
2·5	19·1	14	24
3·5	11·6	6·5	12
4·5	7·0	10	10
5·5	4·3	3	4
6·5	2·6	5	4
7·5	1·6	2	2
over 8	2·4	8	5
Mean	2·00	2·65	2·45

It is, we believe, possible to indicate the cause of the small systematic excess of variance in this exceptionally fine body of data. As has been observed, the duplicate counts, which are recorded in full, do not agree very closely, and it is possible that what may be called "error of counting" is responsible for the existing discrepancy. If we consider such a typical pair of duplicate counts such as that shown in Table XXI, we may regard

Table XXI

Plate	First count	Second count	Difference	Departure from mean
1	70	68	+ 2	+ 8
2	61	72	− 11	− 5
3	54	63	− 9	− 3
Mean			− 6	

the mean difference, as due to the personal equation of the analyst; and the departures from the mean as made up of the several "errors of counting" of the set. If the standard "error of counting" is σ, then the mean value of the sum of the squares of the three departures will be $4\sigma^2$. In this way the standard "error of counting" was estimated for each of the main groups of observations in Series C, divided according to the mean number of colonies per plate, and the additional variance ascribable to "errors of counting" expressed as a percentage of the expected variance.

Table XXII

Percentage variance due to "errors of counting"

Colonies per plate about ...	36	62	82	161	364	All
Increased variance per cent. . .	16 %	24 %	13 %	17 %	59 %	22 %

The effect is thus seen to be a fairly uniform one, though distinctly more prominent among the more crowded plates, of which eight pairs of triplets were available. The higher value in the second group is perhaps due to the fact that these contain the counts of the mixed bacterial population in normal milk, while the others are counts of a practically pure culture of *B. coli*.

The effect ascribable to "errors of counting" is thus of just the right magnitude to explain the additional variance observed in Series C. Since all the groups are affected similarly and nearly to an equal extent, we may anticipate that if this explanation is correct, the actual values of Series C will fit the theoretical expectation if a uniform allowance of 20 per cent. is made for the additional cause of variation. The distributions are so compared in equal intervals of χ^2 in Table XXIII, and by sextiles in Table XXIV.

Table XXIII

χ^2	Expectation with 20 % allowance	Observed
·6	51·9	47·5
1·8	31·5	35·5
3·0	19·1	21
4·2	11·6	12
5·4	7·0	5
6·6	4·3	5
7·8	2·6	3
9·0	1·6	—
over 9	2·4	3

Table XXIV

$\chi^2 = 7{\cdot}545$, $P = {\cdot}185$ ($P = {\cdot}584$)

χ^2	Expectation m with 20 % allowance	Observed $m + x$	x^2
0	22	14	64
·4378	22	28	36
·9732	22	24	4
1·6634	22	21	1
2·6366	22	28	36
4·3003	22	17	25
Total	132	132	166

The distribution shown in Table XXIII shows a remarkably close agreement with expectation. A more exact test of agreement is afforded by the division at the sextiles (Table XXIV); the actual figures show but a moderately good fit with $\chi^2 = 7{\cdot}545$, and $P = {\cdot}185$; since however

duplicate counts of the same plates have been taken as independent observations, χ^2 has been increased by this cause to some extent short of doubling, so that we may say that in reality χ^2 lies between 3·77 and 7·54, while P lies between ·584 and ·185; neither value could be taken as indicating a significant departure from expectation.

We believe, therefore, that in this material, at all events in Series C, the somewhat severe conditions under which the Poisson Series is produced, were in reality fulfilled, and that the departure of the observations from expectation could have been eliminated had precautions been taken to secure a sufficiently accurate counting of the colonies. It must however be borne in mind that the material employed consisted in nearly all cases of almost pure cultures of *B. coli* in milk. The case cannot therefore be compared closely to the different problem of counting such a mixed bacterial flora as occurs in soil, where many different types of organisms, whose growth may be mutually harmful, occur on the plates.

The interference on the plates between dissimilar organisms cannot here be seen, neither can the capability of the medium to check this interference be studied. In this material, for example, there would be little danger of frequent interference by "spreading" organisms, whose growth, had they occurred, would probably have been stimulated by such a medium as was used, containing peptone and meat extract.

The lessened accuracy in counting a mixed flora on this medium is illustrated in Table XXII, where the second group of platings, which contains counts of uninoculated milk, shows a noticeably higher variance in counting than the adjoining groups made from milk cultures of *B. coli*.

The data show, however, that when such a simplified flora is studied, an agreement between parallel platings comparable with the expectations of random sampling can be obtained.

(D) *The data of W. Owen* (14)

One of the most remarkable bodies of data which we have examined is that provided by W. Owen in his investigation of various culture media for the counting of micro-organisms in cane sugar products. In this work, a variety of different media were employed, varying in composition, reaction and osmotic pressure. These were tested in counting bacteria from a variety of sugar refinery products. From the variety of media employed, and from the fact that most of them were new and of untested value, it was to be expected that a rather high variance between parallel platings would be found over the whole series taken together. Had this been the case, separate tests would have been needed of the

indices of variance on the separate media. In fact, however, no such remarkably high variance was found.

The analyses were performed with sets of six plates, and we have chosen the first 100 of these sets for examination. The expected and observed numbers are shown in Table XXV.

Table XXV

χ^2	Expected	Observed	Expected 43%
·5	3·7	38	1·6
1·5	11·3	15	4·9
2·5	14·9	6	6·5
3·5	15·0	9·5	6·5
4·5	13·4	6	5·8
5·5	11·0	3·5	4·8
6·5	8·5	3	3·7
7·5	6·4	3	2·8
8·5	4·7	1	3·5
9·5	3·4	1	
10·5	2·4	—	
11·5	1·7	1	
12·5	1·1	2	
13·5	·8	—	3·4
14·5		—	
15·5	1·6	1	
over 16		10	

The excess of highly variable sets occasions no surprise; we have met with this feature in about the same proportion in Cutler's data. What is astonishing in this case is the immense excess of sets less variable, and in the majority of cases much less variable, than would be the case under undisturbed conditions of random sampling.

In the fourth column we have shown the expected distribution fitted to the total number in the range from 2 to 14. This seems to agree with the distribution observed within this range. We are unwilling to lay much stress on this explanation since the agreement is based on only 36 observations. If it were accepted it would imply that the conditions which lead to the Poisson Series were really operative in about 44 per cent. of the cases, that in at least 10 and probably 11 per cent. excessive variability has been produced, and in the remaining 45 per cent. the variability has been abnormally depressed.

The extent to which the differences between the counts of parallel plates is diminished seems to put the phenomenon beyond the reach of the ordinary explanations; there are some indications, for example, that the plates have not been in all cases completely counted, but it is

difficult to imagine that this cause could be responsible for any such bias as is observed, in view of the fact that a probable error is calculated separately from each set. Severe competition between colonies on the plate is admittedly a possible cause of diminished variability, but we cannot imagine it acting with such severity as would be necessary to explain these results, especially as in the 38 cases in which χ^2 is less than one, the mean number of colonies per plate is always less than 100, and in 15 cases is less than 10.

In more than one instance all the six plates have an equal number of colonies; in samples from a Poisson Series, this would occur but very rarely. For 13 colonies on each plate for example, as is recorded in one instance, the most favourable assumptions will only allow such a coincidence once in some 25,000 trials. Since in the majority of these counts we clearly are not dealing with undisturbed conditions of random sampling, the point cannot be pressed further. We do not agree, however, with the statement that, when such a coincidence occurs, the probable error is zero.

In reviewing the foregoing data, it seems probable that the action of liquefying bacteria, and the development of rapidly growing organisms, unchecked by the medium employed, were the main causes of excessive variance between parallel platings in the work of Buddin and Engberding respectively.

It appears, however, that the conditions of accuracy, such that the development of colonies on parallel platings will form a Poisson Series, can be fulfilled in dealing with a simplified bacterial flora (Breed and Stocking), and have been approached in dealing with the mixed micro-flora of soil, where the medium used has been so devised as to check the excessive development of spreading organisms, as in the case of Thornton's medium. It is possible that these conditions of accuracy would be fulfilled with greater certainty in the case of a mixed micro-flora, if the medium could be further improved so that it checked the growth of such harmful organisms as that found in the Leeds soil (p. 345).

Conclusions

(1) Under ideal conditions the bacterial counts on parallel plates will vary in the same manner as samples from a Poisson Series. When these conditions are fulfilled the mean count of a number of plates is a direct measure of the density of the bacterial population considered (though not, of course, of the total bacterial flora); and the accuracy of such an estimate is known with precision.

(2) For any considerable body of records of sets of parallel plates, agreement with this theoretical distribution may be tested by means of the index of dispersion

$$\chi^2 = \frac{1}{\bar{x}} S(x - \bar{x})^2,$$

where $\bar{x}$ is the mean, and x any individual number of colonies counted on a plate (see Section 5).

(3) From an examination of several large bodies of data we conclude that accurate conformity with the theoretical distribution, though rare, is not unattainable. In particular with a carefully improved technique, and a relatively simple bacterial flora, we believe that the conditions have probably been fulfilled by Breed and Stocking; secondly, by the aid of a specially adapted medium Cutler and Thornton have shown that these conditions have been accurately reproduced, in the great majority of cases, even with the mixed bacterial flora of the soil.

(4) *Any significant departure from the theoretical distribution is a sign that the mean may be wholly unreliable.*

(5) Excessive variance may be produced by the occurrence of certain soil organisms, which have been isolated, and which exert a toxic influence on other forms, and in one case disturb the counts by multiple colony formation.

(6) Subnormal variance is in our experience indicative of some defect in the composition of the medium.

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